CHAPTER

17

Myelin-Associated Glycoprotein Gene

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MAG INTRODUCTION

Specialized glial cells, oligodendrocytes in the central nervous system (CNS), and Schwann cells in the peripheral nervous system (PNS) elaborate cytoplasmic wrappings known as myelin around axons. The myelination process requires a complex series of interactions between the glial cells and axons, which remain poorly understood. Myelin functions to insulate neurons and facilitates the rapid signal conduction required in organisms with complex nervous systems. Myelin-associated glycoprotein (MAG) is a relatively minor constituent of both CNS and PNS myelin that has been implicated in the formation and maintenance of myelin. However, it is also a cell recognition molecule involved in neuron-glial interactions, including regulation of axonal outgrowth and nerve regeneration.

Discovery of Myelin-Associated Glycoprotein, MAG

Prior to the discovery of MAG in 1973, the major proteins in compact myelin such as myelin basic protein (MBP) and proteolipid protein (PLP) were known. During the early 1970s it became clear that proteins on the surface which mediate adhesion are generally glycosylated. Consequently, Quarles and colleagues used radiolabeled fucose to identify MAG (Quarles *et al.*, 1973), a myelin glycoprotein that might mediate adhesive interactions between glial and neuronal cells that are important for the formation of the myelin sheath. MAG was cloned in 1987 (Arquint *et al.*, 1987), and DNA sequence analysis revealed that the MAG cDNA that was isolated was derived from the same mRNA as clone p1B236, a randomly selected, brain-specific, partial cDNA isolated previously in 1983 (Sutcliffe *et al.*, 1983). Originally, 1B236 was thought to be a neuronal protein; however, subsequent studies confirmed that MAG and 1B236 are one and the same (Arquint *et al.*, 1987; Lai *et al.*, 1987a; Noronha *et al.*, 1989). Subsequent findings, described here, have yielded insight into the structure and function of MAG, the major CNS myelin glycoprotein.

Nomenclature

While antibodies (Ab) consist of domains that belong to the immunoglobulin (Ig) superfamily (IgSF) and are involved in protein-protein interactions, there are several Ig-like proteins that recognize carbohydrates and that are also known as I-type lectins (see the review by Kelm, 2001). Cell surface receptors that bind sialic-acid were distinguished as a subfamily of I-lectins soon after the discovery of sialoadhesin, a macrophage adhesion molecule that recognizes sialylated glycans (Crocker *et al.*, 1994). Sialoadhesin was found to share sequence similarity with the CD22 B-lymphocyte surface receptor and also with

MAG. All of these IgSF members bind sialic acid (Freeman *et al*., 1995; Kelm *et al*., 1994), and it was subsequently suggested they be referred to as the ''siglec'' family, derived from sialic acid-binding/immunoglobulin-like/lectin (Crocker *et al*., 1998). There are now 11 members, which are grouped according to their structural and functional similarities (Fig. 17.1). MAG, which is designated siglec-4a, and the related siglec-4b, which is known as Schwann cell myelin protein (SMP) and found in birds, are two of only three siglecs expressed by cells outside of the haematopoietic system. A third siglec with marked sequence similarity to siglec-10, named siglec-11, was recently cloned and found to be expressed on brain microglia (Angata *et al*., 2002). However, there is no mouse ortholog of siglec-11, and hence it is believed to have evolved after the split of primate and rodent lineages. Most of the scientific community continues to use the term MAG instead of siglec-4a, and hence for convenience we do the same here.

MAG Localization

MAG expression is almost exclusively associated with glial cells that will form myelin. Immunolocalization studies at the light and electron microscope levels demonstrate that MAG in the CNS and PNS is found on the Schwann cell and oligodendrocyte membrane in the periaxonal space (Bartsch *et al*., 1989; Martini and Schachner, 1986; Sternberger *et al*., 1979; Trapp and Quarles, 1982). The periaxonal space is the 12 to 14 nm interface between the axon and innermost myelin layer. The electron microscopy (EM) images shown in Figure 17.2A reveal that in young post-natal day (P) 10 oligodendrocytes that have just begun to ensheath axons, anti-MAG Abs label the cell surface (cell body and processes). In developing nerves undergoing myelination, MAG is present in the loosely wrapped myelin layers before the compaction process has occurred. Thus, MAG may function to mediate glial-neuron as well as glial-glial interactions.

In axons that have been fully myelinated, strong MAG immunoreactivity is localized to the periaxonal region and at the inner mesaxon, as seen in Figures 17.2B and 17.2C. However, no MAG is present within the layers of compact myelin. The light microscopy images in Figure 17.3 show the distribution of MAG in cross sections and longitudinal views of peripheral nerve (Figs. 17.3A and 17.3C, respectively); corresponding schematics appear in Figures 17.3B and 17.3D to help distinguish the distribution pattern of MAG and also to identify various features of myelinated nerve. In the PNS, but not in the CNS, MAG is also found in the paranodal loops, Schmidt-Lantermann incisures (channels within the myelin sheath), and there is some low expression at the external (abaxonal) surface of myelinating Schwann cells (Owens and Bunge, 1989; Trapp and Quarles, 1982; Trapp *et al*., 1989b). The functional significance of these differences is not known, although they may reflect different mechanisms used to target MAG to the myelin membrane (Trapp *et al*., 1989a).

Expression of MAG or even contact with a neuron is not sufficient to induce glial cells to form myelin. Cultured oligodendrocytes are able to elaborate myelin-like membrane whorls in the absence of neurons (Bradel and Prince, 1983; Rome *et al*., 1986). Schwann cells require neuronal contact in order to initiate expression of the major myelin proteins such as MAG and MBP; however, these proteins can be readily detected in oligodendrocytes cultured in the absence of neurons (Dubois-Dalcq *et al*., 1986; Owens and Bunge, 1989). Moreover, MAG has been detected on Schwann cells prior to the onset of myelination (Owens and Bunge, 1989; Trapp, 1988), suggesting an additional role before myelination. Evidence is accumulating that transcription factors are involved in controlling whether a glial cell will form myelin, a process that is speculated to be regulated by molecular signals originating from nerves (see reviews by Wegner, 2000; Jessen and Mirsky, 2002). However, the nerve-derived signals and glial signaling mechanisms involved in myelination remain obscure.

Consistent with the notion that MAG also has additional roles besides myelin formation and maintenance is the fact that MAG is expressed in perisynaptic Schwann cells, glia that cover motor nerve terminals at neuromuscular synapses and do not form myelin wrappings (Georgiou and Charlton, 1999). The perisynaptic Schwann cells also express other myelin-related molecules, including the glycoprotein known as protein zero (P0) over most of their membrane. These glia express MAG at their adaxonal membrane and MAG expression persists

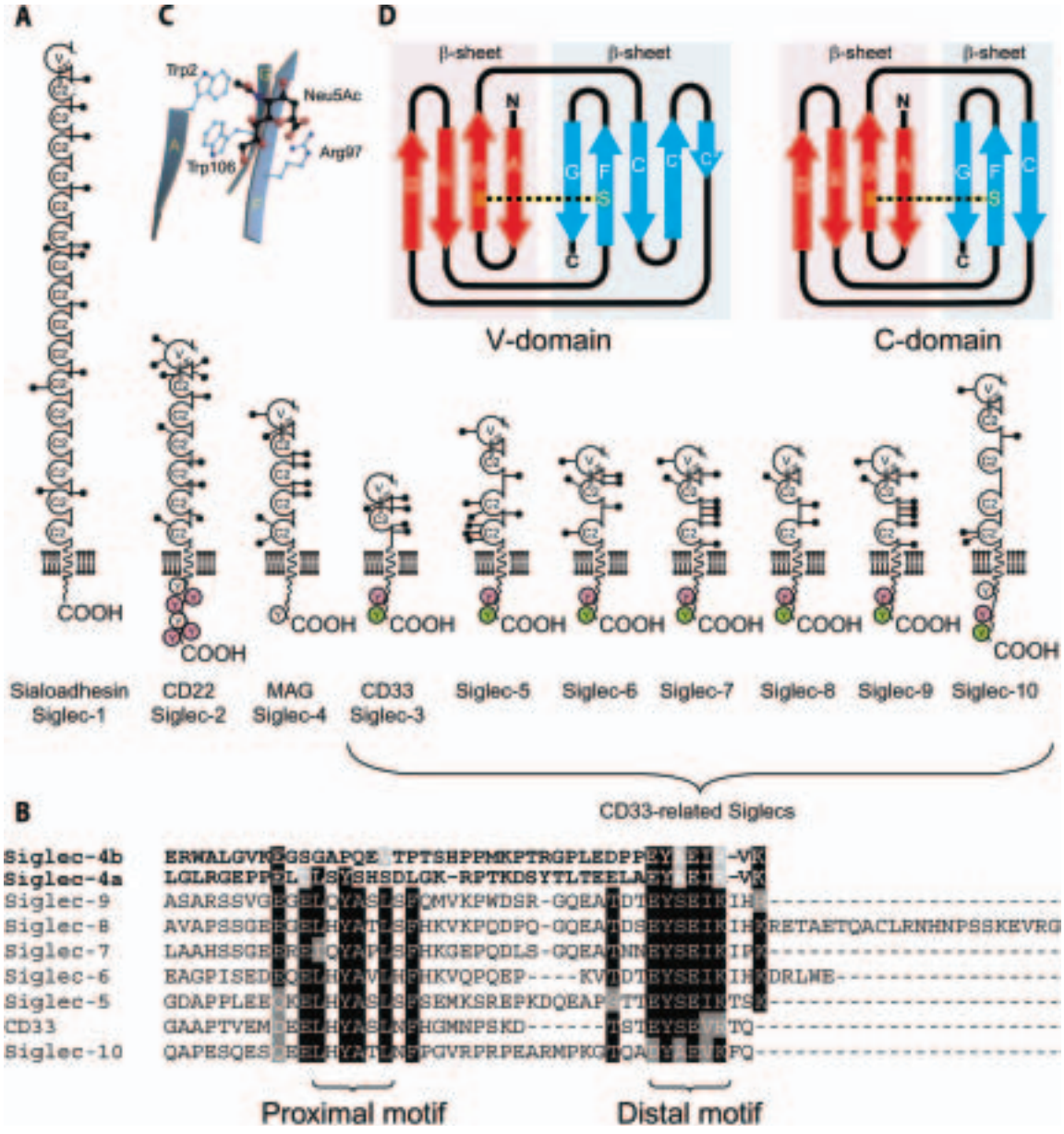

FIGURE 17.1

Structure and comparison of siglecs. Schematic showing Ig-like lectins that bind to sialic acid (siglecs). MAG belongs to the siglec-4a group, together with the related Schwann cell myelin protein (SMP, siglec-4b) found in birds. Not shown is the recently cloned human siglec-11, which has no rodent homolog. (A) Siglecs are type-I membrane proteins with an extracellular region containing a homologous V-set Ig-like domain and a varying number of C2-set Ig-like domains at the N-terminus. The cytoplasmic tails of all siglecs apart from sialoadhesin contain tyrosine residues (Y) within potential signaling motifs. Those motifs that fit the consensus sequence for an immunoreceptor tyrosine-based inhibition motif (ITIM) are shown in pink. The membrane-distal tyrosine-based motifs that are highly conserved in CD33-related siglecs are shown in green. Several siglecs undergo alternative splicing, but only the known full-length forms are illustrated. Potential N-linked glycans are indicated in ball-and-stick form. (B) Alignment of the C-terminal portions of the cytoplasmic tails of CD33-related siglecs reveals two conserved tyrosine-containing motifs. The sequences for siglec-4a and -4b, shown at the top, reveal that the distal motif is similar to the CD33-related siglecs, however, the proximal motif is not conserved. Residues that are identical are boxed in black and residues that are conserved are boxed in gray. The membrane-proximal motif conforms to the consensus ITIM sequence, whereas the distal motif does not. (C) Positions of key residues in sialoadhesin that bind the N-acetylneuraminic acid (Neu5Ac) portion of 3′ sialyllactose, as revealed in a ligand-bound crystal structure of the sialoadhesin N-terminal domain. An essential arginine (Arg97) on the F strand (conserved in the other siglecs) forms a salt bridge with the carboxylate of sialic acid (Neu5Ac) and two tryptophans (Trp2 and Trp106) on the A and G strands form hydrophobic contacts with the N-acetyl and glycerol side groups of Neu5Ac, respectively. (D) Schematic diagram of the V- and C-type domains from immunoglobulins, showing the topology of the β-strands in the two β-sheets. Ig domains belonging to the C2 set have a topology similar to the C-type domain. In domain 1 of sialoadhesin, the inter-sheet disulphide bridge connecting strands B and F is replaced by an intra-sheet dishulphide bridge connecting strands B and E; the C" strand is replaced by a coiled structure and G-strand is split in two. Parts A through C have been modified with permission from Crocker and Varki, 2001, TRENDS in Immunology 22, 337-342, 2001, Elsevier Science Ltd.

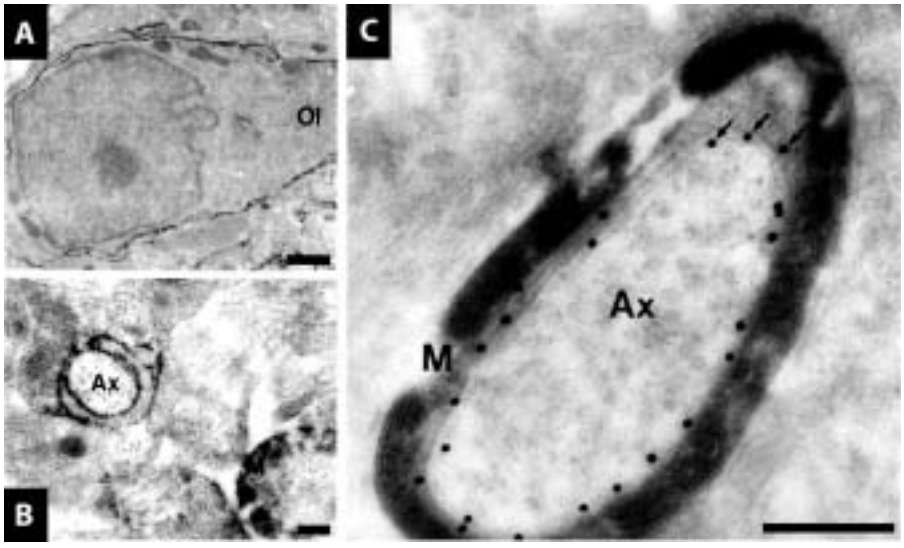

FIGURE 17.2
Electron microscopy reveals MAG's periaxonal localization. Detection of MAG by immuno-EM from mouse optic nerve at P10 (A-B) and P14 (C). (A–B) MAG immunoreactivity on the cell surface (A) and processes (B) of an oligodendrocyte prior or during the time of axon (Ax) ensheathment. Preembedding staining procedures were used in association with anti-MAG Abs and visualized by peroxidase-coupled protein A. (C) When compact myelin surrounds the axon, MAG is confined to the periaxonal region and noncompacted myelin (arrows). Post-embedding staining technique is necessary to demonstrate the periaxonal localization of MAG due to presence of compact myelin (M). MAG was detected with secondary Abs absorbed to colloidal gold (gold particles appear as black dots). Scale bars: A = 1 μm; B, C = 0.2 μm. Reprinted with permission from Bartsch, Kirchhoff, and Schachner, 1989, *Journal of Comparative Neurology* **284,** 451–462, 1989, Wiley-Liss, Inc.

after denervation including at newly formed glial cell extensions. It is possible that factors present at synapses may prevent myelination, or alternatively, MAG may mediate adhesion between axons and surrounding glia. Regardless, it is clear that expression of myelin-related proteins, including MAG, does not obligate glial cells to form myelin wrappings.

Various observations, including MAG adaxonal localization, have suggested that it mediates interactions between the axon and myelin sheath. Expression of MAG on oligodendrocyte tips prior to contact with axons (Bartsch *et al*., 1989), and the fact that MAG is the first myelin protein exported to the tips, further supported a role for MAG in the initial stages of myelin formation. The myelination process begins when the glial cell process (mesaxon) begins to spiral around the axon, and initial observations in the PNS indicate MAG can be first detected in the periaxonal space after 1.5 turns of the mesaxon around the axon (Martini and Schachner, 1986). This suggests that MAG is involved in some aspect of the wrapping process, and as discussed later in the section titled "Multiple Gene Knockouts That Include Deletion of MAG," MAG's involvement in the spiraling of myelinated Schwann cells has also been implicated from studies of double mutant mice lacking P0 and MAG. Finally, MAG has an important role in the control of axonal outgrowth, and this feature is discussed later in the section titled "Control of Axonal Growth and Regeneration by MAG."

Here we introduce that MAG exists primarily as two isoforms, and that the expression of each is regulated temporally and spatially. The isoforms result from alternative splicing to yield a relatively shorter protein known as small MAG (S-MAG) and a longer protein called large MAG (L-MAG). L-MAG predominates during CNS development, including the myelination process, whereas S-MAG accumulates in later stages (Inuzuka *et al*., 1991; Lai *et al*., 1987a; Pedraza *et al*., 1991; Tropak *et al*., 1988). In the peripheral nervous system, L-MAG is always a minor constituent. MAG isoforms have unique signaling capacities, for instance, L-MAG can activate Fyn kinase, and thus the differential expression of MAG isoforms likely has important functional consequences. More information on the unique roles of each MAG isoform will be revealed throughout this chapter.

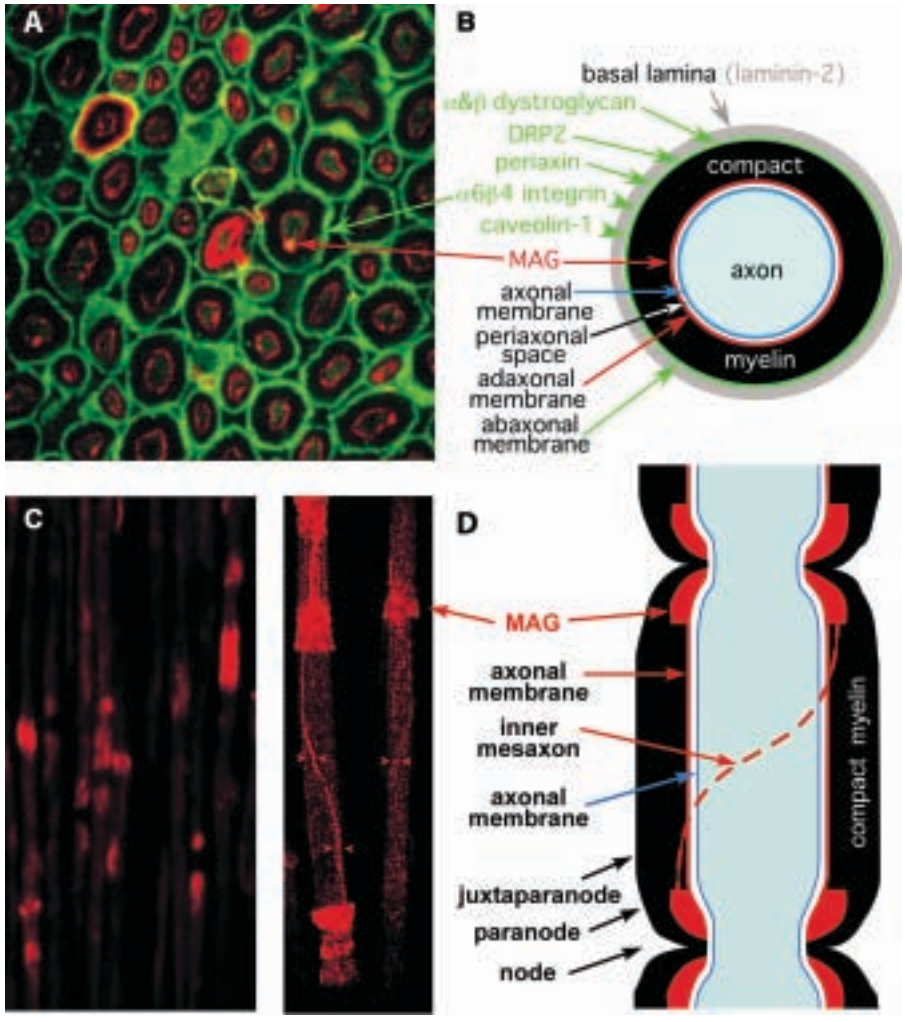

FIGURE 17.3
MAG distribution in myelinated nerve sections. (A) Confocal image from a transverse section of rat sciatic nerve, double-labeled with a mouse monoclonal Ab against MAG (red, detected by TRITC fluorescence) and also with rabbit antiserum against β4 integrin (green, detected by FITC fluorescence). MAG is localized on the inner/adaxonal membrane, and β4 integrin is localized around the entire circumference of the outer/abaxonal membrane. Compact myelin is not stained and thus appears black. (B) The circumferential organization of a myelinated axon is shown schematically. (C) Image on the left shows MAG immunofluorescence from a longitudinal section of sciatic nerve. Image on the right shows MAG distribution in teased single fibers. (D) Schematic showing myelinated axon longitudinal organization and MAG localization. Parts A and B were reprinted with permission from Scherer and Arroyo, 2002, *Journal of the Peripheral Nervous System* **7,** 1–12, 2002 Peripheral Nerve Society, Inc. Part C was modified with permission from Altevogt, Kleopa, Postma, Scherer, and Paul, 2002, *Journal of Neuroscience* **22,** 6458–6470, 2002, by the Society for Neuroscience.

MAG Gene Structure

MAG Promoter and Gene Regulation

Similar to myelin genes such as P0 and P2 basic protein (Peirano *et al.*, 2000), the MAG promoter lacks a TATA box and instead consists of a GC-rich region (Ye *et al.*, 1994). Identification of consensus sequence sites for SP1 and AP2 transcription factors commonly found at GC-rich promoters is consistent with foot-printing experiments using the MAG promoter transiently expressed in an immortalized glial cell line (Laszkiewicz *et al.*, 1997). The regulatory region of MAG extends to either side of a 152 bp promoter core from −1.6 to +0.6 kb. A promoter proximal segment that contains sites for strong transcriptional activators was identified; however, there is a region downstream containing inhibitory cis-elements (Grubinska *et al.*, 1994). The fact that CpG islands in the MAG regulatory region become

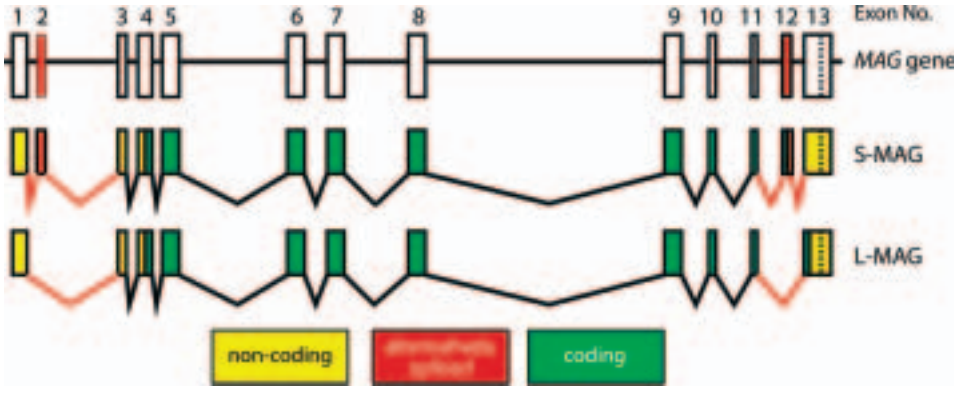

FIGURE 17.4
Schematic of MAG gene locus. The MAG gene contains 13 exons spanning 16 kb and each of its Ig domain is encoded by a single exon (exons 5 to 9). Exons (rectangles), introns (line) and the exons that undergo alternative splicing are indicated (see legend). Exon 12 can be alternatively spliced in, or skipped, to produce S-MAG or L-MAG mRNA transcripts, respectively. There is also alternative splicing of exon 2, which occurs independently of exon 12 splicing (not all combinations are shown); however, it does not contribute to the coding region and its significance is unknown. Alternative polyadenylation cleavage site is depicted as a vertical dashed line in exon 13.

more demethylated as oligodendrocytes progress along the differentiation pathway underscores the importance of methylation in regulation of MAG expression. Transcription from the MAG promoter in Schwann cells does not appear to be affected by glial transcription factors Krox-20 (Topilko *et al.*, 1994) or Sox-10 (Stolt *et al.*, 2002). Although MAG expression is increased in oligodendrocytes in the presence of both transcription factors Oct and Myc, it is not clear whether this is a direct effect (Jensen *et al.*, 1999).

MAG Exon Structure

The MAG gene has been localized to human chromosome 19 (Barton *et al.*, 1987), and mouse chromosome 7 (Barton *et al.*, 1987; D'Eustachio *et al.*, 1988), followed by high-resolution mapping to 19q13.1 (Spagnol *et al.*, 1989; Trask *et al.*, 1993). Elucidation of the intron/exon structure of rat and mouse MAG genes revealed 13 exons spanning approximately 16 kb where each Ig domain is encoded by a single exon (Lai *et al.*, 1987a; Nakano *et al.*, 1991). Exon 12 can be alternatively spliced in, or skipped, to produce S-MAG or L-MAG, respectively. The schematic shown in Figure 17.4 illustrates the structure and products of the MAG gene. Exon 2 from the 5′ noncoding region can also be spliced (Fujita *et al.*, 1989; Lai *et al.*, 1987a; Tropak *et al.*, 1988). Splicing of exon 2 and exon 12 occur independently. One form of MAG lacking exon 2 is predominant in the PNS, whereas mRNA containing exon 2 predominates in the CNS. In contrast to the known differences in signaling capabilities associated with L-MAG versus S-MAG, the significance, if any, of exon 2 splicing is unknown.

Alternative Splicing Produces Two Main MAG Isoforms

Initially, the S- and L-MAG isoforms detected in the CNS were attributed to differential glycosylation (Quarles *et al.*, 1973). Subsequently, the two isoforms were shown to be derived from different mRNAs (Frail and Braun, 1984; Salzer *et al.*, 1987; Tropak *et al.*, 1988) by alternative splicing of exons from a primary RNA transcript (Lai *et al.*, 1987a). The two isoforms of 67 and 72 kDa (sizes of proteins after deglycosylation) share a common region in their cytoplasmic domain but differ in the length and sequence of the amino acids at the C-terminus. The cytoplasmic domain of MAG is encoded by exons 11, 12, and 13. When exon 12 is included, a premature in-frame stop codon results in the shorter S-MAG isoform with an intracellular sequence of 90 residues that contains a unique 10 amino acid C-terminal end. When exon 12 is excluded, the larger L-MAG is produced, which has an additional 54 amino acids derived from exon 13.

Differential expression of the two MAG isoforms during development in the PNS and CNS of rat, mouse, and human was verified at the RNA (Frail and Braun, 1984; Frail *et al.*,

1985; Lai *et al*., 1987b; Miescher *et al*., 1997; Tropak *et al*., 1988) and protein levels (Inuzuka *et al*., 1991; Pedraza *et al*., 1991). Developmental regulation may be related to neuronal contact or the local environment because oligodendrocytes in culture express both MAG isoforms (Tropak *et al*., 1988). Interestingly, when L-MAG is constitutively expressed at high levels in Schwann cells, independent of axonal contact, a greater number of axons are segregated compared to control cells, which express MAG only upon axonal contact (Owens *et al*., 1990). This observation strongly supports the adhesive role of MAG prior to the start of myelination. However, the biological relevance of these observations is not clear, since only low levels of L-MAG can be detected in control Schwann cells and during PNS development (Pedraza *et al*., 1991; Tropak *et al*., 1988).

Although myelin formation by oligodendrocytes and Schwann cells appears outwardly similar, the mechanistic details are distinct. However, oligodendrocytes differ from Schwann cells in several respects. Unlike Schwann cells, which can only myelinate a single segment of an axon, oligodendrocytes are able to simultaneously myelinate multiple segments of different axons. Interestingly, there appears to be a correlation between the relative levels of L-MAG and S-MAG expression and the four morphological types of oligodendrocytes (Butt *et al*., 1998). Thus, it is possible that L-MAG may function in the early events of myelination related to the ability of oligodendrocytes to myelinate multiple axonal segments.

Alternative splicing of MAG is regulated by RNA binding proteins known as QKI. The regulation of MAG isoform expression by QKI was discovered from studies of a naturally occurring mouse mutant known as "quaking" (qk) that has an altered qkI gene regulatory region (noncoding). Reduced QKI levels in qk mice alters the developmental expression of the two MAG isoforms in the CNS and results in S-MAG as the major mRNA throughout development, while L-MAG is scarcely expressed (Frail and Braun, 1985; Fujita *et al*., 1990). The phenotype of qk mice is described later in the section titled "Naturally Occurring Mutations Affecting MAG." QKI contains an RNA-binding domain and belongs to the signal transduction and activator of RNA (STAR) family (Ebersole *et al*., 1996; Vernet and Artzt, 1997). Of the many QKI isoforms that exist, the nuclear localized isoform QKI-5 has been shown to regulate alternative splicing of a MAG minigene as well as the myelin genes PLP and MBP (Wu *et al*., 2002). In the proposed model, binding of QKI-5 to the QASE consensus sequence downstream of the 5′ splice site may interfere with recognition of the splice site or the downstream intronic enhancer, thereby resulting in skipping of exon 12 and concomitant production of the L-MAG mRNA.

MAG BIOCHEMISTRY, STRUCTURE, AND ADHESIVE PROPERTIES

Following translation MAG undergoes several modifications including phosphorylation, remodeling of oligosaccharides by sulfotransferases and sialyltransferases, and palmitylation. With the exception of palmitylation, each of these modifications will be discussed in greater detail. Palmitylation has been shown to be important in membrane targeting and activity of nonreceptor tyrosine kinases (reviewed in Resh, 1994). Similarly, palmitylation of haemagglutinin is important for the infectivity of influenza virus, although the mechanism is unclear (Fischer *et al*., 1998). Pedraza and colleagues experimentally verified the initial prediction that MAG is palmitylated by showing that Cys531 residue in the transmembrane domain is the site of palmitylation (Pedraza *et al*., 1990). Based on other systems, palmitylation of MAG may be important for some aspect of membrane targeting.

Post-Translational Regulation of MAG

MAG Phosphorylation

The primary structure of MAG revealed potential Ser, Thr, and Tyr phosphorylation sites within its cytoplasmic domain (Arquint *et al*., 1987; Salzer *et al*., 1987). In CNS myelin and oligodendrocytes, L-MAG is the predominant isoform that is phosphorylated, whereas

S-MAG is the major phosphorylated isoform in PNS myelin and Schwann cells (Afar *et al*., 1990; Agrawal *et al*., 1990; Bambrick and Braun, 1991; Edwards *et al*., 1988, 1989; Umemori *et al*., 1994; Yim *et al*., 1995). L-MAG is phosphorylated *in vivo* and *in vitro* primarily on Ser, but also on Thr and Tyr residues. S-MAG is phosphorylated constitutively only on Ser. In cultured oligodendrocytes, both S- and L-MAG isoforms are phosphorylated on Ser, while in transformed Schwann cells only S-MAG is present and phosphorylated (Kirchhoff *et al*., 1993). Phorbol ester enhances phosphorylation two- to three-fold, suggesting PKC is involved. Recently it was demonstrated that L-MAG is a PKA substrate (Kursula *et al*., 2000).

S-MAG and L-MAG contain potential Tyr phosphorylation sites, and tyrosine kinases can bind and phosphorylate MAG. Phosphorylation of MAG by v-fps and v-src protein-tyrosine kinases has been demonstrated *in vitro* (Afar *et al*., 1990; Edwards *et al*., 1988). Fyn kinase, a member of the Src family tyrosine kinases, interacts with MAG and phosphorylates the Tyr at position 620 found in L-MAG (Jaramillo *et al*., 1994; Umemori *et al*., 1994). In transfected cells, Fyn only associates with L-MAG, not with S-MAG. In brain lysates however, Fyn was co-immunoprecipitated with both forms of MAG, and conversely, each of L- and S-MAG co-immunoprecipitated with Fyn. The lack of Fyn interaction with S-MAG in the expression system was suggested to be due to the lack of additional molecules that normally mediate this interaction in the brain. Another interpretation is that Fyn does not associate with S-MAG in the brain and that the interaction is seen because S-MAG dimerizes with L-MAG.

MAG Glycosylation

Approximately 30% of the mass of MAG is due to carbohydrate (Quarles, 1983), which is consistent with the eight N-linked oligosaccharide addition sites identified in the predicted amino acid sequence of rat MAG (Arquint *et al*., 1987; Lai *et al*., 1987b; Salzer *et al*., 1987). Mutagenesis experiments of the conserved Asn or Ser residues in the glycosylation consensus sequence have shown that each of the predicted glycosylation sites are utilized when expressed in CHO or COS-1 cell lines (Sgroi *et al*., 1996; Tropak and Roder, 1997). The majority of oligosaccharides on MAG are of the complex type; about two-thirds are tri- or tetra-antennary, one-third are biantennary, and few or none are the high-mannose type (Noronha *et al*., 1989). A high proportion of the oligosaccharides are sialylated and sulphated (Matthieu *et al*., 1975; Quarles *et al*., 1983). Although some experimental evidence suggested that O-linked oligosaccharides may be present in MAG (Pedraza *et al*., 1990), these results have been attributed to the presence of N-glycosidases in the batch of O-glycosidase used to test for the presence of O-linked glycans (Salzer, personal communication).

The carbohydrate epitope recognized by the L2/human natural killer (HNK)-1 Ab (Noronha *et al*., 1986) is expressed on MAG (McGarry *et al*., 1983). It is also found on a number of IgSF member cell adhesion molecules (CAMs) in the nervous system such as the neural cell adhesion molecule (N-CAM), L1, P0 (Bollensen *et al*., 1988; Kruse *et al*., 1984), as well as unrelated adhesive molecules such as peripheral myelin-protein-22 (PMP22) and proteoglycans (Snipes *et al*., 1993). The conservation of the epitope throughout phylogeny (Bajt *et al*., 1990) is suggestive of the importance of HNK-1 in adhesion. Abs against the L2/HNK-1 epitope have been shown to perturb astrocyte-neuron cell adhesion, neurite outgrowth and attachment of cells to laminin (Hall *et al*., 1993; Kunemund *et al*., 1988; Riopelle *et al*., 1986). Oligosaccharides expressing the HNK-1 epitope have been used to block P0-mediated cell adhesion (Griffith *et al*., 1992) and cell-cell and cell-substrate interactions (Kunemund *et al*., 1988). Currently, the gp120 receptor on the AIDS virus is the only protein capable of interacting with the HNK-1 carbohydrate on MAG (van den Berg *et al*., 1992a). In the case of rat MAG, the HNK-1 epitope has been localized to oligosaccharides in either domain 4 or domain 5 (Pedraza *et al*., 1995), whereas all oligosaccharides on human MAG have been suggested to express the HNK-1 epitope (Burger *et al*., 1991). The HNK-1 epitope consists of a 3′ sulphated glucuronyl residue found on glycolipids (Chou *et al*., 1986), N-linked oligosaccharides (Voshol *et al*., 1996), and to a limited extent on O-linked oligosaccharides (Ong *et al*., 2002). Sulphation of the

glucuronic acid is critical for binding of the Ab. However, sulphation alone is not sufficient for binding of Abs recognizing the HNK-1 epitope, since most oligosaccharides on rat MAG are sulphated, yet the protein is poorly recognized by the Ab (O'Shannessy *et al.*, 1985). The species-dependent expression (O'Shannessy *et al.*, 1985) of the epitope, as well as the fact that not all MAG molecules express the epitope (Burger *et al.*, 1992; Kruse *et al.*, 1984), suggests that HNK-1 may not play a major role in MAG function. Alternatively, one component of the HNK-1 epitope, such as the sulphate group, may be important in MAG function.

MAG Structure

On the basis of the amino acid sequence derived from the cDNA for rat MAG, the protein was predicted to be a type I membrane glycoprotein consisting of an N-terminal extracellular domain, a single transmembrane domain and a short cytoplasmic domain (Arquint *et al.*, 1987; Lai *et al.*, 1987b; Salzer *et al.*, 1987). These predictions were later experimentally verified (Johnson *et al.*, 1989; Pedraza *et al.*, 1990). The extracellular portion of MAG was predicted to consist of five Ig-like domains, based on the presence of amino acid sequences in MAG, which are conserved among all members of the IgSF. Human and mouse MAG have 98% and 95% amino acid sequence identity, respectively, with rat MAG (Fujita *et al.*, 1989; Sato *et al.*, 1989; Spagnol *et al.*, 1989). SMP, which overall shares 45% amino acid sequence identity with rat MAG, is very likely a quail ortholog of MAG (Dulac *et al.*, 1992).

IgSF

The IgSF consists of a large number of closely related proteins containing one or more domains that are similar in sequence to domains found in Igs (Williams and Barclay, 1988). The proto-typical Ig domain, found in circulating Igs, consists of two anti-parallel β-sheets held together by an inter-sheet disulphide bridge (see Fig. 17.1D and Amzel and Poljak, 1979). Based on the sequences of Ig-like molecules available at that time, the members of the IgSF were divided into three sets based on the size of the linker between the conserved cysteines and the presence of conserved residues characteristic of the variable (V-) domain or constant (C-) domain from Igs (Williams and Barclay, 1988). The variable domain consists of disulphide-linked anti-parallel sheets with four beta strands (referred to as D, E, B, and A), in one face, and five strands (referred to as C, C′, C″, F, and G) in the other (see Figure 17.1D). In contrast, the C-domain consists of two anti-parallel disulphide-linked sheets with four β-strands (referred to as D, E, B, and A) in one face and three strands (referred to as C, F, and G) in the other. Domains with greatest similarity to the V-domain or C-domain were placed in the V- or C1-sets, respectively. Domains which were similar in size to the shorter C-domain and contained conserved residues found in β-strands D and E from the V-domain were placed in the C2-set. Recently, a fourth set, the I-set, has been defined on the basis of the structure of telokin and its close similarity to other members of the IgSF (Harpaz and Chothia, 1994). Members of the I-set have the same number of β-strands found in the C1- and C2-sets and conserved sequences characteristic of V- and C1-sets.

Disulphide Linkage

Domain 1 of MAG is most similar to Ig domains in the V-set (Williams and Barclay, 1988), whereas the other four domains are most similar to Ig domains in the C2-set (Arquint *et al.*, 1987; Lai *et al.*, 1987a, 1987b; Salzer *et al.*, 1987). The V-like domain 1 of MAG is unique in that the Cys pair (Cys42 and Cys100) may form an intra-sheet disulphide bridge (Williams and Barclay, 1988) as opposed to the inter-sheet disulphide bridge encountered in most Igs. A similar intra-sheet disulphide bridge has been identified in domain 2 of the crystallographically determined structure of CD2, another member of the IgSF (Jones *et al.*, 1992). Domains 1 and 2 of MAG each contain an additional Cys (Cys37 and Cys159) near the N-termini of the domains. Experimental evidence suggests that the additional Cys form an interdomain disulphide bridge between domains 1 and 2 (Pedraza *et al.*, 1990).

Modeling studies suggest that Cys37 and Cys159 are sufficiently close to form an inter-domain disulphide bridge (Kursula, 2001).

MAG Is a Member of the Sialic-Acid Binding Family, Siglec

The unique arrangement of conserved Cys residues in domains 1 and 2 in all MAG orthologues is also a distinguishing feature shared by all IgSF members belonging to the siglec family of I-type lectins. The siglec family of proteins including sialoadhesin, CD22, CD33 family all recognize sialylated glycans. Experimental demonstration of MAG's ability to recognize sialylated glycans on cells was first shown by Kelm and colleagues (1994). In addition to the conserved Cys residues, all functional siglecs contain an invariant Arg residue (Arg118 in MAG, Arg97 in sialoadhesin).

The 3D structure of domain 1 from sialoadhesin complexed with sialyllactose has enabled domain modeling studies of MAG and other siglecs (May *et al*., 1998). Although siglec-1 domain 1 does indeed belong to the V-set of Ig domains, it does, however, differ in several respects. First, it possesses, as predicted, an intra-sheet disulphide bridge between strands B to E rather than B to F. Second, the spacing between the two sheets is increased from the usual 5.6–7.4A range to 8.6A. Third, the C″ beta strand, which corresponds to a region of high diversity in the aligned siglec sequences, is replaced with a quasi alpha-helix like coiling strand. Lastly, the G-β strand is split in two and the other region of siglec diversity corresponds to extended BC loop of unknown function.

The overall structure of a complete siglec has yet to be determined. However, based on EM pictures of a proteolytically generated soluble form of MAG (dMAG) (Sato *et al*., 1984), the molecule appears to be folded back on itself with domains 1 and 2 folded back on domains 5 and 4, respectively (Fig. 17.5, and Fahrig *et al*., 1993). The higher order structure of the domains 1 through 5 is also suggested by biophysical studies using a recombinant soluble derivative of rat MAG (Attia *et al*., 1993). Furthermore, this type of arrangement, where the IgSF domains are folded back in a hairpin/horseshoe configuration have been shown in the 3D structures of hemolin and TAG-1/axonin derived by X-ray crystallography (Freigang *et al*., 2000; Su *et al*., 1998) and EM pictures of L1 (Schurmann *et al*., 2001).

Although domain 1 alone from sialoadhesin retains the ability to bind sialylated glycans (Nath *et al*., 1995), the minimum MAG deletion mutant displaying lectin activity consists of domains 1, 2, and 3 (Kelm *et al*., 1994; Meyer-Franke *et al*., 1995). The sialic acid binding site on MAG has been indirectly mapped to domain 1 using a MAG mutant in which the invariant Arg in domain 1 found in all siglecs has been mutated to Ala/Asp (Tang *et al*., 1997a). Deletion mapping studies of the lectin activity and of a conformational epitope recognized by the 513 monoclonal Ab suggest that the three N-terminal domains of MAG are conformationally linked (Meyer-Franke *et al*., 1995). Similarly, domains 1 and 2 of CD22 appear to be conformationally linked (Nath *et al*., 1995). Thus, the N-terminal domains in some siglecs may not fold independently, but may require the presence of additional domains to attain the mature conformation. The fact that domains 1, 2, and 3 appear to be conformationally linked is consistent with EM and biophysical studies, which suggest that the domains in MAG adopt a higher-order structure with the domains folded back on each other (Attia *et al*., 1993; Fahrig *et al*., 1987).

Two of the differences that contribute to binding of the 3′ sialyllactose lies to the GFCC' face of the Ig domain. The conserved residues Trp2 and Trp106 are directed away from the GFCC β-sheet enabling them to hydrophobically interact with the sialyllactose and resulting in the observed increase in inter-sheet distance. Trp2 interacts with the 5′aminoacyl group, whereas Trp106 contacts the glycerol tail of sialic acid. In most Ig domains these two residues are directed between the β-sheets where they contribute to formation of the hydrophobic core. The split G-strand may enable backbone mediated H-bonds with the hydroxyl groups of the sialic acid glycerol tail. The invariant Arg97, which has been shown by mutagenesis to be critically important for sialic acid recognition, forms a salt bridge with the carboxylate of sialic acid. A similar interaction is seen in the 3D structure of an unrelated sialic acid binding lectin (VP1 from polyoma virus) and possibly other bacterial sialic acid binding lectins (May and Jones, 2001).

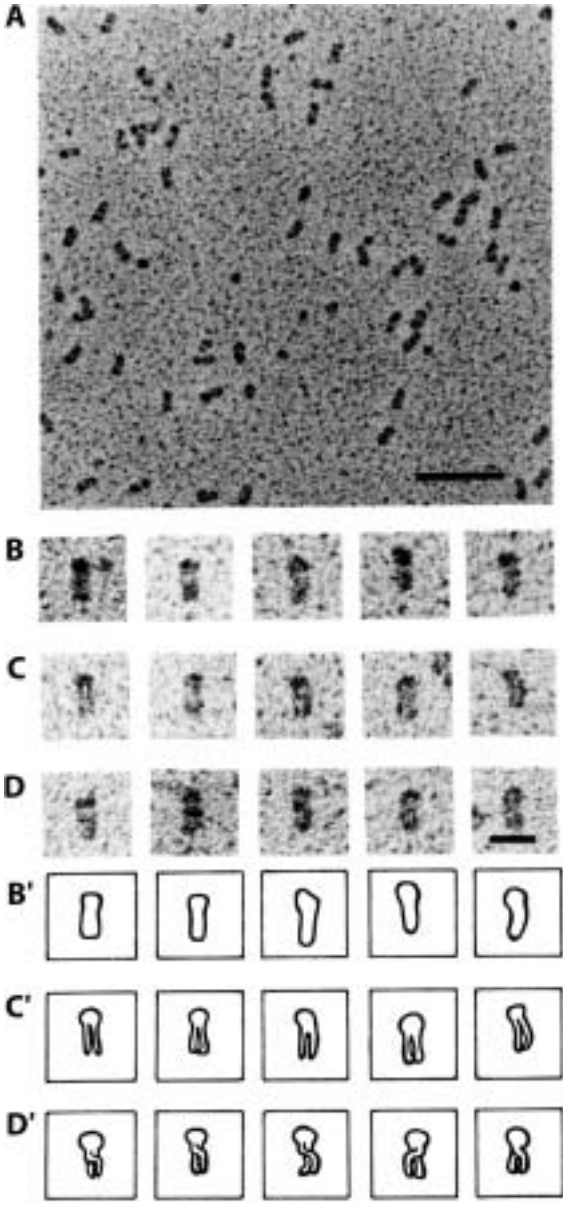

FIGURE 17.5
Transmission electron micrographs of dMAG molecules. Transmission EM visualization by rotary-shadowing technique of soluble dMAG (comprising most of the extracellular part of the molecule, 90 kDa molecular weight). (A) Large field of MAG molecules shadowed with platinum/carbon. (B–D) Selected images of MAG molecules shadowed with tantalum/tungsten. MAG molecules appear as rod-like structures with a globular domain at one end (shown oriented toward top). Depending on the view or transitional state with varied degree of twisting, the nonglobular part of the molecule appears as a compact structure (B), is divided into two thin parallel arms (C) or with an internal thickening (D). (B'–D') Schematic representations of the images shown in (B-D). Scale bars: A = 100 nm; B–D = 25 nm (bar shown in D). Reprinted with permission from Fahrig, Probstmeier, Spiess, Meyer-Franke, Kirchhoff, Drescher, and Schachner, 1993, *European Journal of Neuroscience* **5,** 1118–1126, 1993, European Neuroscience Association.

Structural and Biophysical Features of MAG

All members of the siglec family are type I transmembrane proteins, which have two closely related Ig-like domains belonging to the V and C2-set (Kelm *et al.*, 1994; Powell and Varki, 1995) but differ in the number of additional Ig-like domains belonging to the C2-set. MAG and SMP consist of five Ig-like domains (Dulac *et al.*, 1992). The two isoforms of human and mouse CD22α and β differ in that CD22β contains seven extracel-

lular Ig domains, whereas CD22α lacks Ig domains 3 and 4 (Engel *et al*., 1993; Stamenkovic and Seed, 1990; Stamenkovic *et al*., 1991; Wilson *et al*., 1991). Sialoadhesin has 17 Ig-like domains (Crocker *et al*., 1994). However, cDNAs have been isolated, which suggest the existence of soluble isoforms which are missing C-terminal domains and consist of either 3 or 16 Ig domains. Human and mouse CD33 consist solely of the V and C2-like domains, which define the I-type sialyl lectins (Simmons and Seed, 1988; Tchilian *et al*., 1994). Two isoforms of mouse CD33 have been isolated, which differ in the size and amino acid sequence of the C-terminal tail in the cytoplasmic domain.

In addition to the close similarity of the individual members of the I-type lectins, the localization of CD22 (Wilson *et al*., 1993), CD33 (Peiper *et al*., 1988), and MAG (Barton *et al*., 1987) to a syntenic region of human chromosome 19 suggests that the members have evolved from a common ancestor by gene duplication (Mucklow *et al*., 1995). This prediction is consistent with the fact that the exon boundaries of the CD22, CD33 and MAG (Lai *et al*., 1987b) genes fall within the same regions of the two N-terminal Ig-like domains, and that codons at the boundaries of the exons are in the same phase. Although sialoadhesion is located on a different chromosome (Mucklow *et al*., 1995), this does not necessarily exclude the possibility that sialoadhesin arose from an ancestor common to CD22, CD33, and MAG.

Each member of the siglec family is expressed in a restricted cell lineage and bind preferentially to certain cell types. SMP, which is most similar to MAG (Dulac *et al*., 1992), is expressed on quail Schwann cells (Dulac *et al*., 1988) and oligodendrocytes (Cameron-Curry and Le Douarin, 1995) and is presumed to bind to neurons. CD22, which is expressed on the B-cell lineage (Dorken *et al*., 1986; Engel *et al*., 1993; Stamenkovic and Seed, 1990; Stamenkovic *et al*., 1991), binds to B- and T -lymphocytes and monocytes (Crocker *et al*., 1995; Engel *et al*., 1993; Stamenkovic and Seed, 1990; Stamenkovic *et al*., 1991). In contrast, sialoadhesin is a nonphagocytic receptor, which is expressed on tissue macrophages (Stamenkovic and Seed, 1990; Stamenkovic *et al*., 1991) and binds selectively to neutrophils, although it will also bind to other cells from the granulocyte lineage such as lymphocytes (Crocker *et al*., 1995; van den Berg *et al*., 1992b). CD33 is expressed on the myelomonocytic lineage, such as monocytes and tissue macrophages (Pierelli *et al*., 1993), and selectively binds to cell lines from the myeloid lineage (Freeman *et al*., 1995).

Sialic acids are a family of 9-carbon carboxylic acids usually added to the terminal positions of oligosaccharides by sialyltransferases in the Golgi apparatus (Schauer, 1982; Varki, 1992). Sialyltransferases form a family of proteins, which transfer sialic acid from the donor, CMP-sialic acid, to a specific oligosaccharide acceptor on glycolipids or glycoproteins (Corfield *et al*., 1982; Datta and Paulson, 1995; Paulson and Colley, 1989). Each sialyltransferase catalyzes the attachment of sialic acid via an α-ketosidic linkage to different positions on a monosaccharide unit of a specific disaccharide acceptor. Individual members of the siglec family preferentially recognize sialylated glycans produced by a specific sialyltransferase. CD22 preferentially recognizes sialylated glycans produced by Galβ1-4GlcNAc α2,6 sialyltransferase (2,6N ST), which catalyzes the addition of sialic acids linked via an α2,6 linkage to Gal(β1,4)GlcNAc (2,6N), a disaccharide acceptor commonly found on N-linked oligosaccharides (Kelm *et al*., 1994; Stamenkovic *et al*., 1991). In contrast MAG (Kelm *et al*., 1994), sialoadhesin (Crocker *et al*., 1991; Kelm *et al*., 1994) and CD33 (Freeman *et al*., 1995) recognize sialylated glycans produced by either the Ga1β1-3GalNac α2,3 sialyltransferase (2,3-N ST) or Galβ1-3(4)GlcNAc α2,3 sialyltransferase (2,3-O ST) which catalyze the addition of sialic acid via an α2,3 linkage to Galβ1-3 GalNAc (2,3O) commonly found on O-linked oligosaccharides or Galβ1-3(4)GlcNAc (2,3N) commonly found on N-linked oligosaccharides, respectively. MAG preferentially binds sialic acid containing proteins and glycans, especially the NeuAc α3 Gal β3 GalNAc structure, which is commonly found on gangliosides (Collins *et al*., 1997b; Crocker *et al*., 1996; Kelm *et al*., 1994).

The ability of MAG and siglecs to bind sialylated glycans on the surface of cells represents not only a mechanism for cell adhesion but also a means for regulating adhesion. Thus, the adhesive functions of MAG (Tropak and Roder, 1997), CD22 (Sgroi *et al*.,

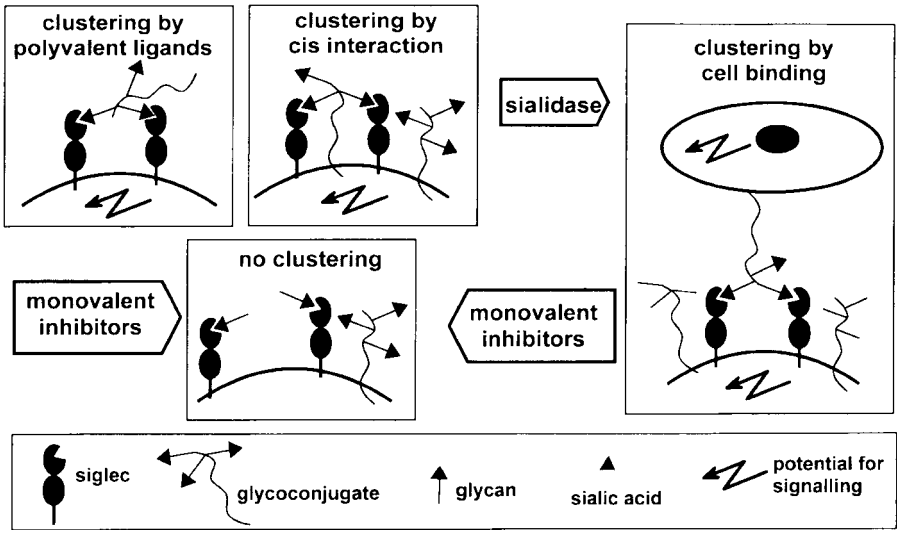

FIGURE 17.6
Potential binding partners of siglecs and their potential role in signal transduction. Potential binding partners for siglecs occur on cell surfaces and in the extracellular space. Siglecs can be clustered on cell surfaces by interacting with multivalent binding partners including cell surfaces, extracellular matrix, or soluble glycoproteins. Intracellular signaling cascades can then be activated, such as the tyrosine phosphorylation of MAG and CD22 cytoplasmic domains that occurs upon clustering of these siglecs. Siglecs can interact with glycoconjugates on the opposing cell (trans-interactions) or also with molecules on the same cell (cis-interactions). Binding of siglecs to trans-ligands could also dissociate the siglec molecules from their cis-binding ligands and influence the signaling properties of these ligands. Binding partners of siglecs on an opposing cell can be clustered through a sialic acid-mediated interaction and subsequently start a signal cascade in the opposing cell. All these events depend on clustering of siglecs, which usually have a greater affinity for multivalent ligands. Monovalent sialosides can also bind to siglecs and inhibit the interaction with multivalent ligands. Reprinted with permission from Kelm, 2001, *Results and Problems in Cell Differentiation* **33,** 153-176, Paul R. Crocker, ed. Mammalian Carbohydrate Recognition Systems, 2001, Springer-Verlag Berlin Heidelberg.

1996), CD33 (Freeman *et al*., 1995), and sialoadhesin (Barnes *et al*., 1999) can be masked by high levels of sialylation in the cell expressing the siglec. One interpretation of these findings is that siglecs are unable to bind to sialylated trans-ligands on the apposing cell because its lectin binding site is occupied by a cis-ligand present in the cell on which the siglec is expressed (summarized in Fig. 17.6). However, sialoadhesin activity is less susceptible to cis-sialylation, presumably due to its size enabling its binding site to be presented above the sialylated glycocalyx surrounding the cell. In the case of CD22, cis-sialylation may prevent inappropropriate activation of the B-cell receptor (Cyster and Goodnow, 1997). The lectin activity of CD22 is unmasked once B-cells enter the lymph nodes where the B-cell receptor may interact with foreign antigens rather than self-antigens.

MAG Adhesive Functions

The adhesive function of MAG was demonstrated experimentally using several different approaches. Poltorak and colleagues first showed that binding of MAG-expressing mouse oligodendrocytes to mouse neurons could be blocked using anti-MAG polyclonal and monoclonal Abs (Poltorak *et al*., 1987). Subsequent incorporation of purified endogenous or recombinant rat MAG into fluorescently labeled liposomes demonstrated binding to in vitro cultures of rodent dorsal root ganglion (DRG) neurons (Johnson *et al*., 1989; Poltorak *et al*., 1987). Figure 17.7 shows fluorescently labelled liposomes containing recombinant S- or L-MAG, in which L-MAG and S-MAG bound primarily to neurons in spinal cord cultures, whereas only the S-MAG liposomes bound to DRG neurites. MAG liposome binding to neurons was blocked by anti-MAG monoclonal Ab 513 (Figs. 17.7E through 17.7F) and can also be blocked by monoclonal Ab 15 (Meyer-Franke

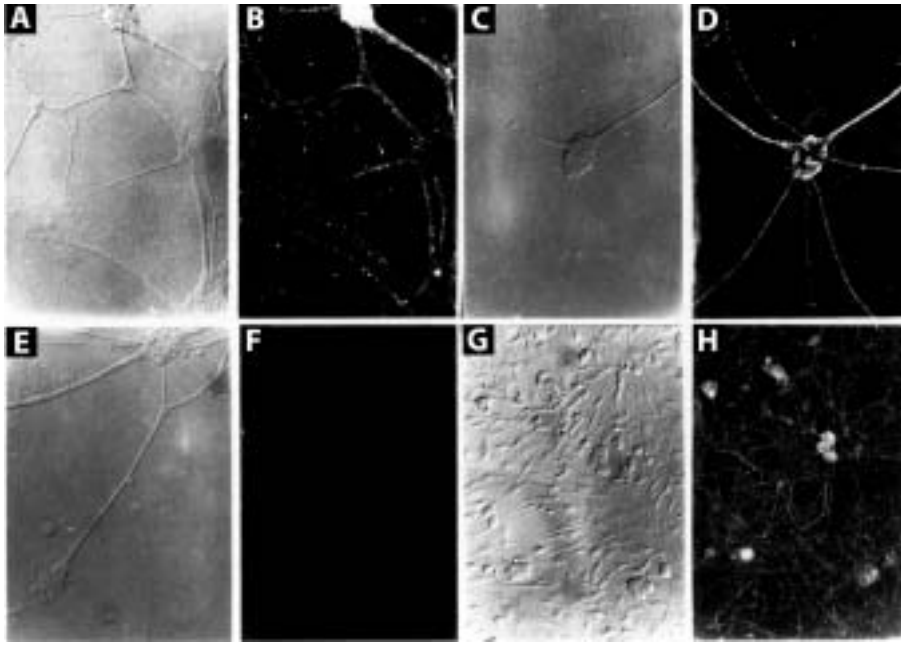

FIGURE 17.7
Neural Adhesion of Recombinant MAG proteins. One-week-old DRG cultures from newborn mice or 4-week spinal cord cultures from P13 embryonic mice. Liposomes labelled with carboxyfluorescein and containing recombinant S- or L-MAG were incubated for 30 minutes with cultures, washed, then visualized under optics to reveal phase (A, C, E, and G) and also fluorescence (B, D, F, and H). (A–B) Spinal cord cultures incubated with L-MAG liposomes. (C–D) Spinal cord cultures incubated with S-MAG liposomes. (E–F) Spinal cord cultures were incubated with monoclonal anti-MAG 513 Ab (Fab fragment) and S-MAG liposomes. (G–H) DRG cultures were challenged with S-MAG liposomes. Modified with permission from Johnson, Abramow-Newerly, Seilheimer, Sadoul, Tropak, Arquint, Dunn, Schachner, and Roder, 1989, *Neuron* **3,** 377–385, 1989 by Cell Press.

and Barres, 1994). MAb 513 was raised against glycoproteins immunopurified from chicken brain membranes using the L2/HNK-1 Ab (Poltorak *et al*., 1987). In contrast, the IgM rat MAb 15 was raised against immunopurified mouse MAG (Meyer-Franke *et al*., 1995). Initial experiments showed that MAG only bound to DRG neurons, which could be myelinated, but not to cerebellar neurons, which are not myelinated *in vivo*. Thus, MAG was thought to interact only with a neuronal ligand, which was found on axons destined to be myelinated. Subsequent experiments demonstrated that MAG liposomes could in fact bind to cerebellar neurons, possibly as a result of the different culture conditions used to grow the neurons (Sadoul *et al*., 1990). Furthermore, it was shown that MAG could mediate the heterophilic aggregation of L cell fibroblasts (Afar *et al*., 1991), CHO cells (Attia, 1992), and oligodendrocyte-like cells (Almazan *et al*., 1992). It is unlikely that these cells expressed a neuronal ligand. These results experimentally verified the prediction that MAG could function as a CAM. However, these observations also suggested that MAG could recognize a number of different ligands.

As has been demonstrated for most CAMs (see the review by van der Merwe and Barclay, 1994), the interaction of MAG with its ligands appears to be multivalent. Thus, neither an engineered soluble form of MAG nor the proteolytically generated soluble form of dMAG is able to bind to neurons (Attia, 1992; Sadoul *et al*., 1990). However, dMAG is able to bind with high affinity (K_D ~10^{-7} M) to various types of collagen and heparin (Fahrig *et al*., 1987; Probstmeier *et al*., 1992). The inability of MAG liposomes to bind to these ligands suggests that the manner in which MAG is presented (i.e., soluble versus on the cell surface) may affect binding to collagen. Although the biological significance of this type of interaction is not clear, it may be indicative of MAG's ability to bind to different ligands.

MAG interacts with several extracellular matrix molecules. MAG binds to specific types of collagen and by so doing reduces collagen fibril formation and also integrin-mediated

adhesiveness of neural cells (Bachmann *et al*., 1995; Probstmeier *et al*., 1992). The ability of MAG to modify constituents of the extracellular matrix suggests it plays a role in controlling adhesive interactions and recognition between cells.

MUTATIONS AFFECTING MAG

Naturally Occurring Mutations Affecting MAG

The earliest indication of MAG's role in mediating interactions between the axon and myelin sheath was provided by studies in the dysmyelinating recessive mouse mutant known as ''quaking'' (Sidman *et al*., 1964). The mutation that accounts for the phenotype in the quaking mouse (qk) occurs in the 5′ regulatory (noncoding) region of the qkI gene, which generates three alternatively spliced transcripts encoding the QKI proteins QKI-5, -6, and -7, putative RNA binding proteins suggested to link RNA metabolism with signal transduction (Ebersole *et al*., 1996; reviewed by Hardy, 1998). Compared to wild-type mice, QKI protein levels are reduced drastically in myelinating cells of qk mice (Hardy *et al*., 1996). In this mutant, there is an increased molecular weight of both MAG isoforms due to abnormal glycosylation (Bartoszewicz *et al*., 1995; Matthieu *et al*., 1974). However, in qk mice, the expression of L-MAG is greatly reduced both at the RNA (Frail and Braun, 1985; Fujita *et al*., 1988) and protein levels (Bartoszewicz *et al*., 1995; Bo *et al*., 1995; Fujita *et al*., 1990). Another factor that contributes in reducing L-MAG levels in the qk mice appears to be increased endocytosis of L-MAG from periaxonal membranes (Bo *et al*., 1995). Regarding S-MAG expression in qk mice, there is an inverse effect in that the relative levels are increased (Frail and Braun, 1985; Fujita *et al*., 1990). The modified L- and S- MAG levels in qk mice are a result of altered QKI expression. For instance, a recent study found that QKI-5 regulates the alternative splicing of MAG by repressing the inclusion of exon 12, which is skipped in L-MAG and included in S-MAG (Wu *et al*., 2002). The loss of QKI-5 in qk mice may thus be responsible for the alterations in MAG splicing, although it is not known whether the lack of QKI-6 and -7 also contribute.

Myelin sheaths of qk mice are disrupted in regions lacking MAG. Immuno-EM studies revealed that in regions of the periaxonal space where MAG cannot be detected, the characteristic 12 to 14 nm space between the axonal membrane and Schwann cell membrane is altered (Trapp *et al*., 1984). Furthermore, in the periaxonal region where MAG was absent, the thickness of the periaxonal cytoplasmic collar (PCC) is reduced. Normally, myelinating glia from wild-type mice have a well-developed PCC that spans more than half of the axonal circumference. Although loss of regional expression of MAG is correlated with the previously stated morphological deficits, the expression of other myelin proteins, such as MBP, is also affected in qk (Li *et al*., 2000). Therefore, it is possible that the altered expression of other proteins besides MAG may account for the loss of the periaxonal collar in quaking.

B. MAG knockout mice reveal a mixture of minor myelin defects

MAG-deficient mice reveal various subtle defects related to myelin formation and maintenance (also see the review by Schachner and Bartsch, 2000). MAG null mice have been generated by two separate groups using gene targeting methods (Li *et al*., 1994; Montag *et al*., 1994). These knockout mice have defects in myelin that are not severe and largely affect CNS myelin sheaths. The modest changes observed in MAG null mice is in contrast to the observed inability of Schwann cells to segregate large caliber axons and form myelin *in vitro*, when MAG expression was greatly reduced (more than five-fold) using MAG antisense RNA (Owens and Bunge, 1991). Although MAG expression in the Schwann cells was reduced but not eliminated, it is not clear whether this can account for the difference between the *in vitro* and *in vivo* experiments. The phenotype is probably not due to secondary effects of using MAG antisense RNA because Schwann cells infected with the MAG sense virus formed normal compact myelin.

Mutant mice that express a truncated form of the L-MAG isoform have also been developed and these highlight the disparate importance of L- and S-MAG isoforms in the

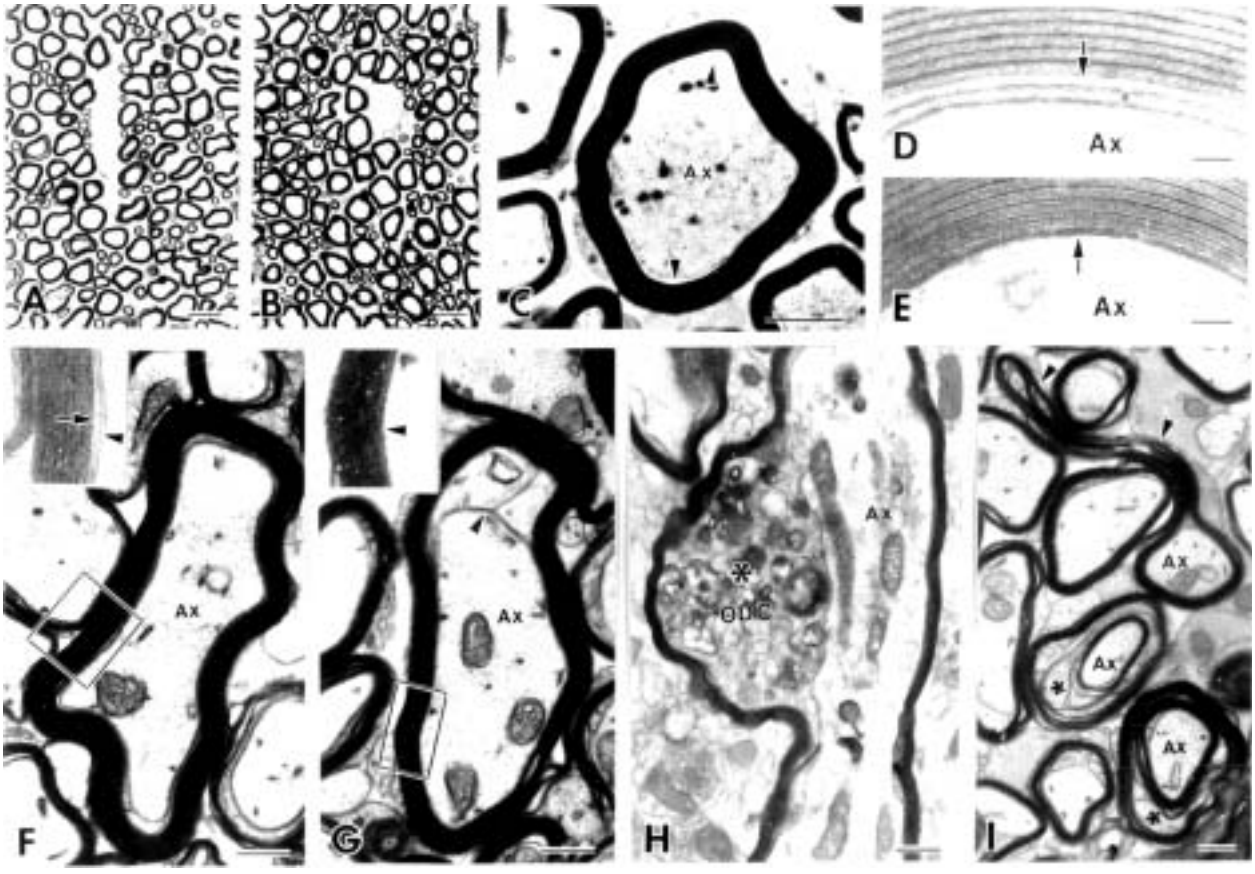

FIGURE 17.8

MAG knockout mice have subtle changes in myelin morphology. Myelin morphology in PNS (upper panels) and CNS (lower panels) of MAG wild-type (+/+) and knockout (−/−) mice. (A–B) Light micrograph sections of L2 ventral spinal roots do not reveal any striking differences in myelin or neurons between MAG +/+ and −/− mice, respectively. (C) Electron micrograph (EM) of a myelinated axon (Ax) from the L4 ventral root of a MAG −/− mouse, displaying a dilated periaxonal space (arrowheads). (D–E) EMs from L2 ventral root of MAG +/+ and −/− mice, showing loss of the normal 12 to 14 nm periaxonal space (asterisk) and the Schwann cell cytoplasmic collar (arrow). (F–G) EMs from optic nerve fiber of MAG +/+ and −/− mice reveal that the normal periaxonal space (see insert, arrowhead) and cytoplasmic collar (insert, arrow) are reduced or missing in oligodendrocytes from a knockout animal (except in the mesaxon region, where the cytoplasmic collar is present, arrowhead). (H) Longitudinal section of a MAG −/− optic nerve fiber showing an oligodendrocyte (ODC) with focal disorganization of the periaxonal cytoplasmic collar (asterisk). (I) Transverse section of MAG −/− optic nerve fibers showing disrupted compact myelin lamella (asterisks) and redundant compact myelin (arrowheads). Scale bars: A,B = 10 μm; C, F, G, H, I = 0.5 μm; D, E = 0.05 μm. Reprinted with permission from Li, Tropak, Gerlai, Clapoff, Abramow-Newerly, Trapp, Peterson, and Roder, 1994, *Nature* **369,** 747–750, 1994, Macmillan Publishers Ltd.

CNS and PNS, respectively (Fujita *et al*., 1998). An emerging theme from studies of double knockout mice is that compensation by MAG-related molecules occurs in single mutants, and thus it has been difficult to fully assess the normal role of MAG *in vivo* based on analysis of MAG mutant mice alone. Nonetheless, important details on MAG function have emerged.

Delayed CNS Myelin Formation in MAG-Deficient Mice

Myelin formation is delayed in the CNS, but not PNS, of mice possessing a null mutation in the MAG gene. Compared to wild-type mice, only half as many retinal ganglion cell axons are covered by compact myelin in 10 day old MAG mutants and optic nerves of adult MAG nulls contain more unmyelinated and small-sized axons (Bartsch *et al*., 1997; Li *et al*., 1998; Montag *et al*., 1994). Surprisingly, however, myelination in the PNS of MAG knockouts proceeds normally and essentially normal compact myelin is formed in both the PNS and CNS of young (<3 months) animals (Li *et al*., 1994; Montag *et al*., 1994). The changes in morphology of myelin in the PNS and CNS of MAG knockout mice are shown in the EMs of Figure 17.8.

Collapse of Periaxonal Cytoplasmic Collar in MAG Null Mice

One of the subtle abnormalities observed in MAG-deficient mice includes collapse of the PCC in the CNS but not everywhere in the PNS (Li *et al*., 1994; Montag *et al*., 1994). The EMs in Figures 17.8F and 17.8G show the presence and absence of PCCs from CNS optic nerve fibers of MAG wild-type and MAG-deficient mice, respectively. This defect is also shown in the EMs of Figure 17.9, which are from another line of MAG knockout mice. Also, as seen in the EMs of Figures 17.8D and 17.8E, Schwann cells from the L2 ventral roots of MAG null mice, but not wild-type mice, frequently lack PCCs (Li *et al*., 1994). It should be noted that abnormal PCCs do not occur in all regions of the nervous system since they are not detected in sciatic and femoral nerves (Fruttiger *et al*., 1995a; Montag *et al*., 1994). Similar to the difference in relative functional importance of MBP in the PNS and CNS, MAG may play a major role in the maintenance of the PCC in the CNS, whereas in the PNS, it seems to play a minor role. The loss of regional expression of MAG is correlated with collapse of the PCC in the PNS of qk mice (Trapp *et al*., 1984); however, the expression of other myelin proteins is also affected in qk and thus the loss of additional proteins besides MAG may account for the loss of the PCC.

Multiple Myelin Wrappings in MAG-Deficient Mice

MAG may regulate CNS myelination via control of glia-axon recognition or adhesion. For instance, MAG-deficient mice were found to have an increased frequency (2- to 50-fold) of axonal segments ensheathed by two or more individual myelin sheaths in young (<3 months old) and especially in mature (>11 months old) animals (Bartsch *et al*., 1995b; Li *et al*., 1994, 1998; Montag *et al*., 1994). Figures 17.8I and 17.9B show examples of retinal ganglion cell axons from MAG-deficient mice that are surrounded by two distinct myelin sheaths. In some cases, up to four myelin sheaths have been observed, which do not necessarily spiral in the same direction around the axon. Related abnormalities include oligodendrocyte cytoplasm within compact myelin sheaths, redundant myelin loops, and also myelinated axons and myelin debris, which are grouped by a large myelin sheath (Fig. 17.8I). While myelination does proceed even in the absence of MAG, glia in the CNS fail to establish when, where, and how much myelin to form.

It is not yet known whether the multiple myelin wrappings are formed during the initial stages of myelination or whether the original sheaths are later displaced from the axon by additional sheaths. Multiply myelinated structures are generated in the PNS when Schwann cells invade an existing myelin sheath and lay down a new sheath underneath the previous sheath (Kidd and Heath, 1988). In the CNS of MAG deficient mice, sequential invasion of preexisting myelin sheaths by oligodendrocytic processes may result in multiply myelinated axonal segments. Thus MAG may play a regulatory role in myelination, possibly by recognizing myelination-competent axons. In fact, thin cytoplasmic membranes from several Schwann cells can normally contribute to form a myelin-like sheath, which surrounds the soma of spiral ganglion neurons, and, interestingly, MAG is only expressed in the adjacent myelin that surrounds axons but not in myelin around the soma (Martini, 1994). The lack of MAG may reduce adhesion between glia and axons and allow multiple glial sheaths to surround axonal membranes.

Glial Cell Abnormalities in MAG Knockouts

Analysis of aged mice lacking MAG unveiled a defect in the long-term maintenance of myelin. While no abnormalities could be detected in the PNS myelin of young (<10 weeks) mice with a null mutation in the MAG gene, degenerating axons and myelin sheaths were observed with increased frequency in the PNS of older (>9 months) MAG knockout mice (Fruttiger *et al*., 1995a; Weiss *et al*., 2001; Yin *et al*., 1998). Morphological analysis of peripheral nerves revealed the presence of onion bulbs, a feature of human peripheral neuropathies, which are formed upon degeneration-induced Schwann cell proliferation and remyelination. The degenerating myelin was often associated with degenerating axons, and it has been suggested that the formation of globular expansions of superfluous myelin, known as myelin tomacula, are responsible for the axonal degeneration.

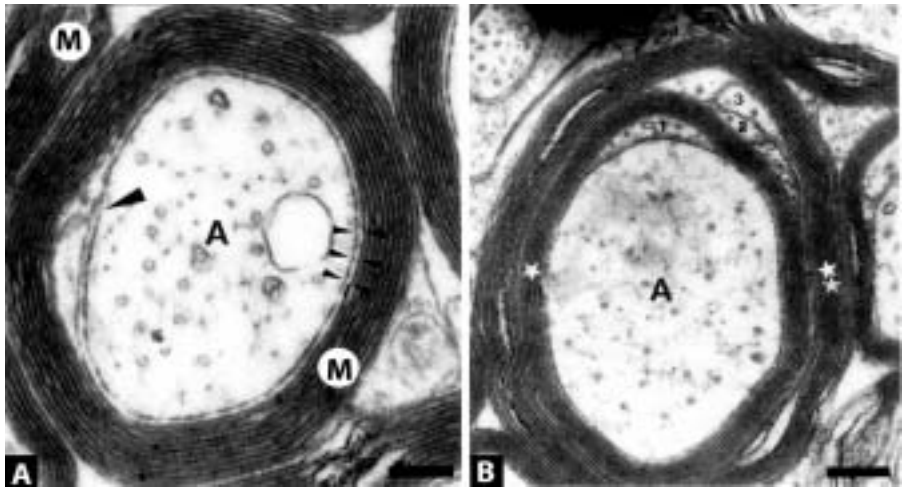

FIGURE 17.9
Multiple myelin wrappings surround axons of MAG-deficient mice. EM cross-sections of myelinated retinal ganglion cell axons (marked with an A) from 2-month-old MAG null mice. (A) Normal appearance of compact myelin (M), periaxonal space (arrowheads), and inner mesaxon (large arrowhead). Note the absence of a periaxonal cytoplasmic collar (compare with periaxonal cytoplasmic collar from wild-type axons shown in Fig. 17.8F). (B) Axon surrounded by two distinct myelin sheaths. Cytoplasm at the inner aspect of the inner myelin sheath (one star) is confined to the inner tongue process (1). The inner myelin sheath terminates in the outer tongue process (2) in the immediate neighbourhood of the inner tongure process (3) of the outer myelin sheath (two stars). Note the separation between the two sheaths. The outer tongue process of the outer myelin sheath (4) appears near the top of the image. Scale bars = 0.2 μm. Reprinted with permission from Montag, Giese, Bartsch, Martini, Lang, Blüthmann, Karthigasan, Kirschner, Wintergerst, Nave, Zielasek, Toyka, Lipp and Schachner, 1994, *Neuron* **13,** 229–246, 1998 by Cell Press.

An infrequent CNS abnormality that occurs more often in older MAG null mice involves changes in the structure and function of oligodendrocytes. It is not known how early the changes can be detected; however, oligodendrocytes from 3-month-old MAG-deficient mice begin to show signs of a disorganized cytoplasm with accumulation of organelles (Fig. 17.8H; also see Li *et al*., 1994). By 9 months of age, oligodendrocyte processes show a dystrophy that resembles dying-back oligodendrogliopathy (Lassmann *et al*., 1997; Weiss *et al*., 2000), commonly found in diseases of toxic or immune-mediated demyelination. Some of the defects that develop in the cytoplasm of oligodendrocyte processes include deposits of vesicular material, multivesicular bodies, mitochondria, and lipofuscin granules. The dying back process is believed to represent a response to injury, which does not involve oligodendrocyte apoptosis. The underlying mechanism is not known, but it could involve a loss of MAG-mediated signaling from myelinated axons to oligodendrocytes. The oligodendrocyte dystrophy in MAG nulls may be related to the similar pathology that occurs in multiple sclerosis (MS) and other demyelinating diseases. It is important to note that the oligodendroglial pathology occurs before any signs of demyelination.

Altered Morphology of Axons in MAG Null Mice

Axons in MAG knockout mice have a reduced caliber and more densely packed neurofilaments (Yin *et al*., 1998). Cross-sections of myelinated axons from MAG null mice reveal that the 10-nm thick dots corresponding to neurofilaments are spaced closer together, compared to axons from wild-type mice (Fig. 17.10). In unmyelinated fibers of control mice and MAG-deficient mice, neurofilament spacing is identical. Thus, the reduced neurofilament spacing correlates with the loss of MAG in the adaxonal Schwann cell membrane. Myelin itself is not responsible for regulating neurofilament spacing because animals that cannot form myelin have increased axon diameters and normal spacing of neurofilaments (Sanchez *et al*., 1996). Although it is discussed later on in the section titled ''Autoimmune Neuropathy,'' we briefly mention here that neurofilament spacing is

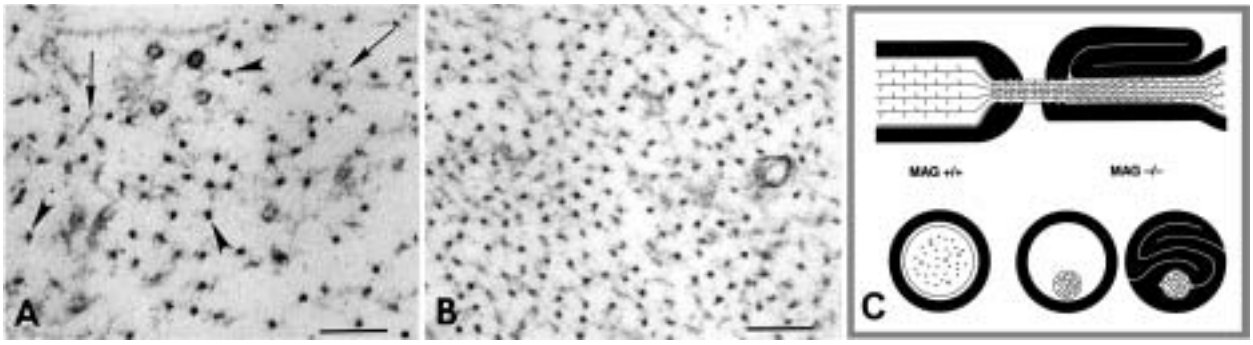

FIGURE 17.10
MAG-deficient axons have reduced neurofilament spacing and axon caliber. (A) and (B) Electon micrographs showing neurofilaments, which appear as 10 nm round dots (arrowheads), in cross sections of sciatic nerve from 9 month old control mice and MAG-deficient mice, respectively. Arrows point to thread-like sidearms that extend from neurofilaments. Note the wide spacing between neurofilaments in axons from control mice. Scale bars: A, B = 0.1 μm. (C) Schematic representation of axon-myelin sheath structure from the paranodal region in longitudinal (C1–C2) and transverse (C3–C5) sections. In axons from wild-type mice (C1 and C3), the paranodal region contains neurofilament, which is spaced apart and correlates with large axonal caliber. Paranodal regions from MAG nulls (C2, C4, and C5) are surrounded by tomaculi, and neurofilament spacings and axonal calibers are similar to those in nodal regions. Reprinted with permission from Yin, Crawford, Griffin, Tu, Lee, Li, Roder, and Trapp, 1998, *Journal of Neuroscience* **18,** 1953–1962, 1998, by the Society for Neuroscience.

reduced in patients with demyelinating neuropathies associated with serum Ab against MAG (Lunn *et al*., 2002). Thus, there is adequate evidence to support a role for MAG in modulating the structure and possibly function of axons. Further work will need to establish whether a lack of MAG impedes nerve cell function.

Biochemical Changes in MAG-Deficient Mice

MAG null mice have alterations in expression of other proteins, which are summarized in Table 17.1. Levels of the Schwann cell protein CNP (2′, 3′-cyclic nucleotide 3′-phosphodiesterase) are reduced by half in MAG knockout mice compared to control mice (Weiss *et al*., 2000, 2001). Also, expression of N-CAM was reported to be reduced, although other studies described either no changes or an increase (Li *et al*., 1994; Montag *et al*., 1994). There is also decreased expression of certain axonal neurofilaments, which may contribute to changes in morphology described earlier. It is not clear whether any decrease in protein expression is a direct consequence of MAG absence or due to loss of membranes associated with demyelination.

MAG Knockout Mice Have Neurological and Behavioral Defects

In addition to morphological and biochemical changes, there are electrophysiological and behavioral abnormalities in MAG knockout mice. Compared to control animals, aged animals lacking MAG have reductions in sciatic nerve conduction velocity (Weiss *et al*., 2001). However, the changes were not as dramatic as those found in other demylinating neuropathies, and furthermore, there was no significant change in action potential amplitude from which to suggest an axonal neuropathy. The reduction in axonal caliber that occurs in MAG nulls could explain why conduction velocity is impaired. Because MAG is also necessary for long-term maintenance of the myelin sheath, demyelination may also contribute to the electrophysiological abnormality. However, compact myelin is largely unaffected in MAG nulls, which favors the hypothesis that alterations in some aspect of axonal function is responsible.

Mice Lacking L-MAG-isoform

Mutant mice lacking L-MAG have a phenotype similar to that of MAG knockout mice. The mice were made by introducing a stop codon into exon 13 of the MAG gene, which normally encodes for L-MAG containing the longer, carboxyl-terminal, cytoplasmic segment (Fujita *et al*., 1998). The L-MAG mutants produce a truncated L-MAG protein that

TABLE 17.1 Comparison of MAG Knockout Phenotypes

	Genotype	MAG -/-	L-MAG -/-	PLP -/-	MBP -/- shiverer	MAG -/- PLP -/-	MBP -/- PLP -/-	MAG -/- PLP -/- MBP -/-	GalN AcT -/-
BIOCHEMISTRY									
mRNA	MAG	0	only 1-MAG		3.5	0	2.7	0	0.5
	PLP			0		0	0	0	
	MBP	1.5			0		0	0	
	N-CAM 140	1.6							
	N-CAM 120							1.5	
	OMgp	0							
Protein/ganglioside	MAG		0.5 S-MAG			0		0	
	PLP			0		0	0	0	
	MBP	2 (PNS)			0	2 (PNS)	0	0	
	GD1a/GT1b				0		0	0	
	CNP	0.5							
	NF-H, NF-M	~0.5 - 0.7							
	cdk5 and ERK 1/2	~0.6 - 0.7							
MORPHOLOGY									
Glial cells	Myelin	reduced PCC, multiple myelin sheaths, oligo dystrophy (CNS), redundant myelin (CNS/PNS) axon degeneration of old mice with myelin tomacula (PNS)	multiple myelin sheaths, redundant myelin, similar to *MAG* nulls NO myelin and axon degeneration in aged mice	dissociation of outer surfaces, condensed and dissociated IDL	hypo-myelination, reduced microtubule expression and density (CNS) more SLI (PNS)	dissociation and de-compaction of the IDL	hypo-myelination pseudo-myelin (CNS), more SLI (PNS)	hypo-myelination pseudo-myelin (CNS), more SLI (PNS) pseudo-IDL	decreased CNS melination and axonal degeneration (CNS and PNS)
	Number of Glia				more oligos		more oligos	more oligos	
Neuron	Axon degeneration	late onset (PNS)		late onset (PNS)		early onset (CNS)	late onset (CNS)	early onset (CNS)	
	Axon Calibre	reduced							
BEHAVIOR									
water maze	learning (latency)	0.5				0.1			
	mean swim speed								
open field	locomotion	0.5			0.1	0.6	0.6	0.1	
	grooming	0.3-1.1			0.4		0.5	0.4	
	rearing	0.2			0.1	0.6	0.5	0.1	
specialized assays	bar: grooming	0.5							
	rotarod (time)				0.4	0.3	0.4	0.1	
	horizontal wire								
	horiz. bridge (latency)	0.2			0.5	0.3	0.1	0.1	
NEUROLOGY									
	tremor / seizures	mild intention tremor	tremor by P12			tremor by P28	hi-frequency tremor	seizures	
	conduction velocity	only > 12 mo.							
	lifespan (months)	>24		>24	3-4	>24	>24	7-9	

(Continues)

TABLE 17.1 (*Continued*)

Genotype		N-CAM -/-	MAG -/- N-CAM -/-	PO -/-	MAG -/- PO -/-	CGT -/-	MAG -/- CGT -/-	Fyn -/-	MAG -/- Fyn -/-
BIOCHEMISTRY									
mRNA	MAG		0		0		0		0
	PLP								
	MBP								
	N-CAM 140	0	0						
	N-CAM 120	0	0						
	OMgp								
Protein/ganglioside	MAG		0		0		0		0
	PLP								
	MBP								
	GD1a/GT1b								
	CNP								
	NF-H, NF-M								
	cdk5 and ERK 1/2								
MORPHOLOGY									
Glial cells	Myelin	degeneration of axons and myelin	degeneration of axons and myelin occurs earlier than in N-CAM null	hypo-myelination, abnormal myelin compaction	longer delay in myelin formation, abnormal myelin compaction	multiple myelin sheaths, redundant myelin disoriented paranodal loops, periaxonal splitting (CNS)	no PCC, hypo-myelination, multiple myelin sheaths, severely disoriented paranodal loops, periaxonal myelin splitting (CNS)	>50% less myelin (CNS), myelin sheaths normal	severe hypo-myelination, 80% of optic nerve axons lack myelin, also all of same defects found in *MAG* nulls
	Number of Glia								
Neuron	Axon degeneration		earlier onset						
	Axon Calibre								
BEHAVIOR									
water maze	learning (latency)								
	mean swim speed								
open field	locomotion								
	grooming								
	rearing								
specialized assays	bar: grooming								
	rotarod (time)								
	horizontal wire								
	horiz. bridge (latency)								
NEUROLOGY									
tremor / seizures						tremor by P14	obvious tremor, P12		
conduction velocity									
lifespan (months)						3	<1		

Regarding quantitative data, an increase greater than 50% appears in red, a reduction to less than 50% appears in blue, and green signifies no change. These cut-off values are arbitrarily chosen and do not necessarily correspond with differences by statistical significance. Unless noted otherwise, biochemistry reflects analysis of CNS myelin. Much of the data was obtained from tables found in Uschkureit *et al.* (2000) and other articles referenced in the chapter. Acronyms: SLI = Schmidt-Lantermann-incisures, IDL = interperiod dense line

is four amino acids shorter than S-MAG, whereas the endogenous S-MAG isoform is unaffected. CNS axons from L-MAG knockout mice were surrounded by multiple myelin sheaths, there was redundant myelin, and some oligodendrocyte cytoplasm was present in compact myelin. These abnormalities in the L-MAG mutants are similar to and occur at the same frequency as in MAG nulls (13 to 15% of CNS axons). In addition, L-MAG mutants develop a rapid tremor by P12 and tonic seizures in adulthood. Although the total MAG protein level was reduced by half in the L-MAG null mice, the reduced levels are likely not responsible for the observed defects because they do not occur in mice that are heterozygous for the MAG null mutation.

Perhaps more interesting is the observation that older L-MAG mutants do not show signs of myelin and axon degeneration in the PNS. This is in contrast to the marked neuropathological abnormalities seen in aged MAG null mice. The simplest interpretation of the data is that L-MAG is not necessary and that S-MAG suffices for PNS myelin formation and maintenance. The extracellular domain of MAG appears to mediate processes involved in maintaining the integrity of the PNS. In contrast, the longer cytoplasmic domain found in L-MAG is critical for CNS myelin formation.

Multiple Gene Knockouts That Include Deletion of MAG

There has been a tremendous effort to find myelin-related molecules that may functionally compensate for the lack of MAG in MAG knockout mice. Some myelin-related molecules can have overlapping function, and thus deletion of a single gene do not produce many defects. Compensation may sometimes be accomplished by up-regulation of related molecules. For instance, in MAG null mice as well as in MAG/PLP double knockouts, MBP expression is up-regulated twofold in peripheral nerves (Li *et al.*, 1994; Uschkureit *et al.*, 2000). There is also a two- or three-fold overexpression of S-MAG in the CNS of MBP nulls and PLP/MBP double mutants. Even when there are no such changes, the potential for overlapping function is still there, and it has become necessary to produce multiple gene knockouts by cross-breeding candidate single mutants to MAG nulls. This search has excluded a compensatory role for L1 because MAG/L1 double mutants do not have more deficits than what occurs in the MAG knockout alone. Compared to control mice, MAG/L1 double knockout mice have a similar number of myelinated axons (Haney *et al.*, 1999). However, in several other combinations that are reviewed later in this chapter, there are more complex phenotypes, and these have confirmed suspected MAG functions and also revealed some novel functions of MAG.

Axonal Degeneration in Mice Lacking MAG, PLP, and MBP

Studies on double and triple knockouts of MAG in combination with either PLP or MBP have corroborated the belief that MAG has a role in promoting axonal stability (Uschkureit *et al.*, 2000). As an aid to comparing the phenotypes between the various MAG knockouts, a table has been contructed based largely on data from the previously mentioned study (Tab. 17.1). Briefly, PLP is an integral CNS myelin sheath protein, which plays a role in stabilizing the apposition of the adjacent extracellular membrane surfaces. MBP comprises one-third of the CNS and one-tenth of PNS myelin and has an important role in the compaction of the opposing cytosolic surfaces of the plasma membrane processes. In PLP/MAG double knockouts, severe neurological symptoms develop, which is in contrast to their respective single mutants. A tremor of the hindlimbs develops by P28 and progresses to dragging and jerky-like movement with increasing age. Optic nerves from PLP/MAG double mutants show early onset and rapidly progessing axonal degeneration, which temporally parallels the neurological symptoms. Behavioral tests also revealed more severe motor and learning defects in mutant mice lacking both PLP and MAG (see Tab. 17.1). In triple mutant mice of genotype MAG−/− PLP−/− MBP−/−, a high-frequency tremor develops by 3 months after birth and the life span is reduced to about 8 months, in contrast to the PLP/MBP double-mutants that have a normal life span. There is early onset axonal degeneration in the triple mutant, again suggesting that the deprivation of MAG is detrimental to axons. The findings support a role for MAG in maintaining the integrity of CNS axons.

MAG/N-CAM Double Knockouts

N-CAM partially compensates for MAG functions related to maintenance of axon-myelin integrity. While there is some uncertainty as to whether N-CAM expression is altered in MAG knockouts, N-CAM is still present and could take over functions normally carried out by MAG. To examine the extent of possible compensation by N-CAM in myelin formation and maintenace, MAG/N-CAM double knockouts were generated by cross-breeding single mutants (Carenini *et al*., 1997). Myelin formation was not affected in the double mutants; however, degeneration of axons and myelin occurred earlier than in MAG-deficient single mutants. More pathological abnormalities were detected in the MAG/N-CAM double mutants by 4 to 8 weeks of age, although by 26 weeks both mutants were equally affected. Therefore, there was no indication that these molecules are necessary for myelin formation, however, both contribute to maintenance of axon and Schwann cell function. A subsequent study determined that N-CAM and MAG can contribute to myelin formation, although these functional roles are only detectable in the absence of P0.

Delayed Myelination in MAG/P0 Null Mice

The most abundant glycoprotein in PNS myelin is P0, which when absent causes severe dysmelination (Giese *et al*., 1992). Interestingly, P0 contains an Ig-like extracellular domain, which confers recognition ability. MAG/P0 null mice were generated to analyze their combined roles, and the double mutants had abnormal compaction in myelin sheaths that was similar to that in P0 null mice (Carenini *et al*., 1999). However, the number of axon-Schwann cell units devoid of myelin is greater in 10-day-old MAG/P0 double mutants compared to P0 mutants. This indicates that in mice lacking P0, the formation of turning loops of Schwann cell processes depends on the presence of MAG. The role of MAG in promoting the spiraling of myelinating Schwann cell processes cannot be detected in MAG null mice because P0 seems to compensate. In P0/MAG double-deficient mutants, the lack of N-CAM also impairs Schwann cell spiraling. However, the effect is more transient. Thus, MAG has functions in myelin formation that cannot be duplicated fully by other molecules.

MAG/Galactolipid-Deficient Mice Show Defects in Adhesion

MAG- and galactolipid-deficient mice have some common deficiencies, and double mutants were generated to test for compensatory activity. Mice in which the gene for the enzyme UDP-galactose:ceramide galactosyltransferase (CGT) is mutated can no longer convert ceramide to the myelin galactolipids galactocerebroside and its sulfated form sulfatide (Bosio *et al*., 1996; Coetzee *et al*., 1996). CGT mutant mice have a progressive neuropathological phenotype, which includes tremoring by P14, and they die near P86. However, similar to the MAG mutant, myelin formation is largely unaffected and in the CNS of CGT mutant mice, and there are three times as many unmyelinated processes as well as multiply myelinated axons (Dupree *et al*., 1998a, 1998b; Marcus *et al*., 2000). MAG/CGT double mutants have a more severe phenotype than the single CGT mutant with a much more obvious tremor and rapid hindlimb paralysis and reduced life expectancy of 22 days (Marcus *et al*., 2002). The double knockouts have a noticeably absent PCC whereby eight-fold fewer processes retain a normal periaxonal space and eight times more spinal cord fibers display periaxonal splitting. There are additional exacerbations, such as in paranode structure, which we will not discuss here. The report concludes that MAG and myelin galactolipids act synergistically at the paranode to promote its structural maintenance. Also, these molecules functionally overlap with one another in maintaining intercellular adhesion along myelinated axons. While there are no recognized axonal ligands for galactolipids, the authors note that each of sulfatide and MAG have been shown to interact with members of the tenascin extracellular matrix protein family, which could modulate adhesion during myelin formation.

Hypomyelination in MAG/Fyn Double Mutants

The multiple knockout approach has been used to assess whether MAG effects on myelin involve intracellular signaling via Fyn. Fyn is a nonreceptor-type tyrosine kinase of the Src

family, which when absent leads to significant impairment of myelination (Umemori *et al*., 1994). Fyn is found in myelin-forming cells and is activated by stimulation through cell receptors including L-MAG, which subsequently leads to transcription of the MBP gene for myelination (Umemori *et al*., 1999). Compared to wild-type mice, Fyn-deficient mice have only half as much myelin in their brain and even less in spinal cord. To determine whether MAG and Fyn interact in the myelination process, double knockouts were generated by cross-breeding the single mutants (Biffiger *et al*., 2000). Double knockout of MAG and Fyn revealed hypomyelination that is far more severe than that of single mutants. Half of the axons from optic nerve are unmyelinated in Fyn null mice, but this ratio increases to 80% in MAG/Fyn double knockouts, which is greater than the 20 to 30% found in MAG nulls. If MAG and Fyn operate via the same signaling mechanism, then there should not have been a more severe phenotype in the double mutants. However, it is still possible that MAG and Fyn actions are not independent because compensatory mechanisms may exist in the single mutants. For instance, a MAG-associated kinase activity still persists in Fyn nulls (Umemori *et al*., 1994), which could partially compensate for the loss of Fyn.

MAG/Fyn double mutants have the same morphological defects in their myelin sheaths as in MAG null mice. There is redundant myelin, multiply-myelinated axons, degeneration of myelin sheaths, impairments in the oligodendrocyte cytoplasmic collar, and noncompacted regions of myelin. It is important to note that Fyn single mutants do not have any defects in their myelin sheaths, which suggests that Fyn does not participate in the formation of normal, intact CNS myelin. MAG can therefore activate other downstream signaling molecules to produce morphologically intact myelin.

MAG Can Act as an Inhibitor of Axon Regeneration

Finally, we will discuss briefly the inhibitory role of MAG in axonal regeneration, which was demonstrated in crosses of MAG nulls with C57BL/Wld^s mutants. After injury to the PNS of C57BL/Wld^s mutant mice, axon degeneration occurs slowly, as does removal of myelin due to poor leucocyte invasion (Brown *et al*., 1991, 1992; Lunn *et al*., 1989). Together with the observation that Schwann cells with the greatest amount of lesion-induced myelin breakdown are more frequently contacted by regrowing axons (Fruttiger *et al*., 1995b), this suggested that myelin inhibits axonal regeneration. However, offspring generated from crosses of C57BL/Wld^s and MAG null mice show much improved axonal regeneration in regions along Schwann cells with nondegenerated myelin (Schäfer *et al*., 1996). Still, these mutants have even better axonal growth in regions lacking intact myelin, which has been interpreted to indicate that either additional myelin-associated inhibitors exist or that the persistent myelin sheaths can act as a mechanical barrier for sprouting axons. Nonetheless, the presence of MAG in the PNS of C57BL/Wld^s mutant mice partially contributes to inhibit axon growth. It is not known whether this inhibitory activity of MAG has any biological significance, because upon injury to nerves from normal wild-type mice, myelin is removed and Schwann cells down-regulate expression of myelin-related proteins. Thus, MAG is absent in the injured PNS, and its growth-inhibitory function may only be important in the normal adult for limiting aberrant growth. The following section focuses on this inhibitory aspect of MAG, together with the receptors involved.

CONTROL OF AXONAL GROWTH AND REGENERATION BY MAG

During development of the nervous system, axons send out extensions at a rate and direction that is regulated by factors in their environment. When axons reach myelin, their growth is blocked. MAG and several other proteins found in CNS myelin are inhibitory to axon outgrowth and regeneration. We will discuss the functions that MAG plays in mediating inhibition, including the role of recently identified MAG receptors. While the focus will be on axon growth and regeneration, it is important to review these

events because the mechanisms will almost certainly have some relevancy to the process of myelination.

MAG Promotes Axonal Growth during Early Development

Although MAG is a potent axonal growth inhibitor in the adult nervous system, during early development, MAG is not inhibitory. Indeed, most of the founding experiments that studied the role of MAG on neurite outgrowth made use of embryonic and neonatal neurons. MAG increases outgrowth of young cerebellar and spinal cord neurons and also neonatal DRG neurons (Johnson *et al*., 1989; Matsuda *et al*., 1996; Mukhopadhyay *et al*., 1994; Turnley and Bartlett, 1998). However, when post-natal neurons are exposed to MAG, their axonal growth is inhibited. Unfortunately, this developmental switch in the neuronal response to MAG was not realized for quite some time, causing considerable confusion to scientists.

It is not clear whether the neurotrophic-like effect of MAG on young neurons has any biological significance. For instance, axons have stopped growing by the time MAG becomes expressed at detectable levels, and thus MAG is likely not normally involved in the promotion of neurite growth during early development. Nonetheless, a recent investigation has found that the L-MAG isoform is responsible for promoting axonal growth. Specifically, when cerebellar neurons from newborn mice were cultured on BALB/c 3T3 cells transfected with cDNA for either the S- or L-MAG isoform, only the latter had growth promoting activity (Shimizu-Okabe *et al*., 2001). Because L-MAG is the principle form that is expressed during development, it is speculated to function in enhancing axon outgrowth. It remains to be seen whether low levels of L-MAG exist during development or perhaps could assist axonal recovery following injury or disease. Also, by selecting young neurons that are not inhibited by MAG and do not express receptors that contribute to inhibiton of axonal outgrowth (discussed later), it may be possible to use young neurons as an aid for axon regeneration.

In the Adult, MAG Functions as an Inhibitory Molecule

MAG was the first myelin-derived molecule shown to be inhibitory to axonal growth in the adult nervous system. MAG inhibits growth of neurites (including neuronal-like cells) regardless of whether it is presented in soluble forms containing the extracellular domain, is expressed by live cells, or when introduced to axons by way of immobilized membrane prepared from MAG-expressing cells (DeBellard *et al*., 1996; Li *et al*., 1996; McKerracher *et al*., 1994; Mukhopadhyay *et al*., 1994; Shen *et al*., 1998; Tang *et al*., 1997b). Furthermore, Schwann cells that are induced to express MAG also inhibit regeneration, demonstrating that MAG is inhibitory in both the adult CNS and PNS.

Several factors that affect MAG efficacy were not recognized until recently, and this also hindered progress on accepting it is an inhibitory molecule. For instance, neuronal growth cones were found to collapse upon encountering MAG-coated beads (Li *et al*., 1996), whereas another study did not find any such effect using MAG extracted from myelin (Bartsch *et al*., 1995a). However, the inhibitory role of MAG on neurite outgrowth has also been confirmed using MAG fused to the Fc portion of human IgG and also dMAG (Tang *et al*., 1997b). The authors emphasized several important details, which should be considered when studying MAG and which likely explain the seeming discrepancies. For instance, the manner by which MAG is presented can affect activity, in that MAG is multimeric when coated onto beads, compared to its monomeric, relatively denatured format when extracted from myelin and solubilized. Furthermore, the MAG content in purified myelin varies considerably and MAG is also rapidly (within minutes to hours) converted to dMAG, which would be lost upon any washing.

MAG-mediated inhibition of axonal growth can also be overcome by the presence of other proteins and also by neurotrophins. Laminin, a component of the Schwann cell basal lamina, can override the growth inhibitory activity of MAG (David *et al*., 1995). Hence, after purification of MAG from myelin, any residual laminin can nullify MAG inhibition

of neuronal outgrowth. MAG also binds the extracellular matrix glycoprotein tenascin-R which is expressed by oligodendrocytes (Yang *et al*., 1999). Tenascin-R neutralizes MAG-mediated inhibition of neurite outgrowth. Finally, neurotrophins such as BDNF or GDNF have been shown to prevent MAG-mediated inhibition of axonal regeneration (Cai *et al*., 1999).

Upon injury to the adult nervous system, myelin-forming glia will down-regulate MAG. This biological change is of extreme benefit, given the inhibitory attribute of MAG on axonal outgrowth. Indeed, axons in the PNS can regenerate successfully, especially when aided by the additional removal of any remaining MAG by macrophages and also by the induction of regeneration-associated genes. The inhibitory nature of MAG in the PNS has been demonstrated by the finding that axonal regeneration can be improved in C57BL/Wld^s mutant mice (which do not clear myelin and show hardly any axonal regeneration) after crossing them to MAG null mice (as already discussed; also see Schäfer *et al*., 1996). However, axons in the CNS do not grow back, a problem that is of great clinical relevance for injuries and diseases of the nervous system and that has preoccupied researchers for many years.

Myelin in the CNS contains two additional proteins that inhibit neuronal growth. Caroni and Schwab identified two membrane proteins, NI35 and NI250, and also raised IN-1 Abs, which detected these proteins and could counteract the nonpermissive properties of CNS myelin (Caroni and Schwab, 1988a;1988b). NI250 was next purified and shown to inhibit neurite outgrowth (Spillmann *et al*., 1998). Several labs then used peptide sequences of NI250 (now called Nogo-A) to clone the Nogo gene, named for its ability to block axonal growth (Chen *et al*., 2000; GrandPre *et al*., 2000; Prinjha *et al*., 2000).

Nogo codes for three proteins, Nogo-A, -B, and -C, and there is agreement that Nogo-A is inhibitory. All three isoforms share a conserved C-terminal region with two transmembrane domains connected by a 66-amino acid linker sequence known as Nogo-66. The Nogo-66 domain is believed to be on the extracellular side of membranes and is most likely the domain responsible for inhibition of neurite outgrowth. Curiously, the N-terminal region of Nogo-A is also inhibitory, although the mechanism of action is different from Nogo-66 (Chen *et al*., 2000; Fournier *et al*., 2001; Prinjha *et al*., 2000). Also, the amino-Nogo domain is suspected to be cytoplasmic and it may only play a role if released following disease or injury. Further work is necessary to sort out the details of Nogo membrane organization and domain differences in mediating inhibitory actions.

Finally, the last known myelin-derived inhibitor is the oligodendrocyte-myelin glycoprotein (OMgp). OMgp is a glycophosphatidylinositol (GPI)-anchored protein, which remained elusive until very recently when it was identified to bind to the same receptor as Nogo and to inhibit neurite outgrowth *in vitro* (Wang *et al*., 2002). A receptor for Nogo-66, named the Nogo-66 receptor (NgR), was identified by expression cloning using a mouse cDNA library (Fournier *et al*., 2001). NgR was determined to contain a signal sequence followed by eight leucine-rich repeat domains and a cysteine-rich region, followed by a GPI linkage region. The human homologue of the NgR gene has exons (separated by nearly 30 kb) on chromosome 22q11. The NgR cDNA sequence encodes a protein 473 amino acids long with a 85 kDa apparent molecular mass. There is widespread expression in the brain and very little expression in the PNS. In summary, MAG, OMgp, Nogo, and NgR all contribute to the inhibitory activity of CNS myelin.

Identification of Functional Neuronal MAG Receptors

MAG Binds the Nogo Receptor, NgR

Immediately prior to when this chapter was written, several groups made the important discovery of identifying the NgR as a common functional receptor for MAG and OMgp (Domeniconi *et al*., 2002; Liu *et al*., 2002; Wang *et al*., 2002). Figure 17.11 contains experimental data from the article by Liu and colleagues and elegantly demonstrates that MAG mediates inhibition of axonal outgrowth via NgRs. First, NgR levels in DRG neurons are shown to be minimal during embryonic development and to increase into

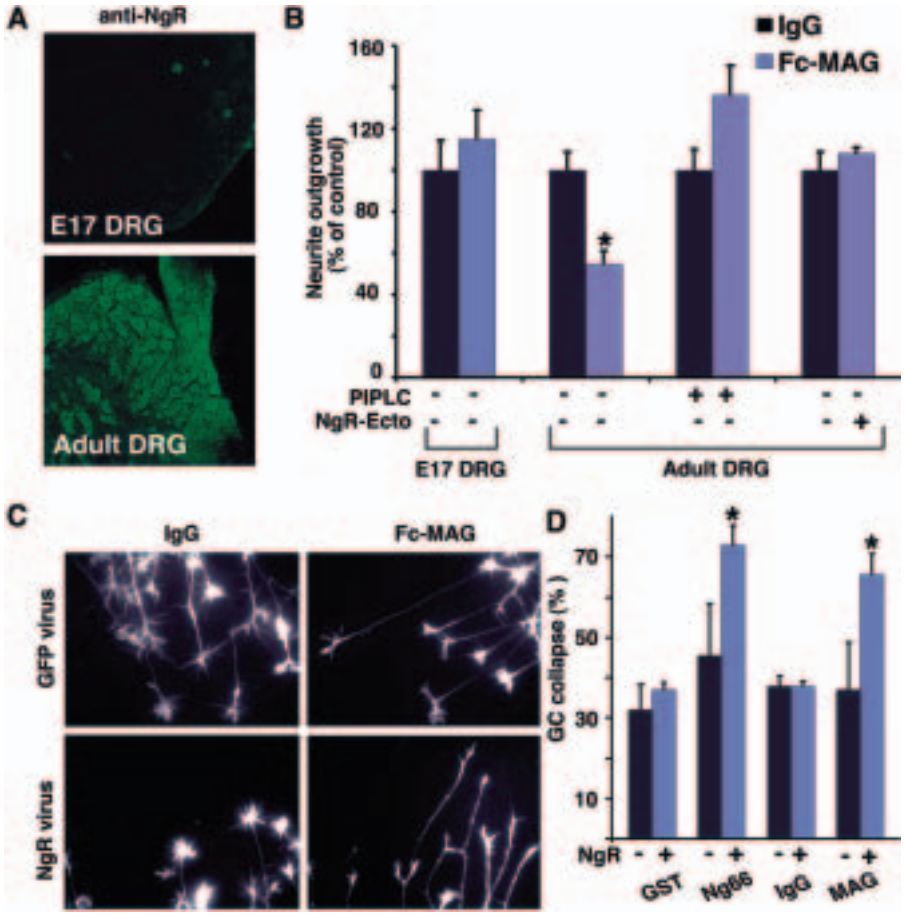

FIGURE 17.11
The Nogo receptor (NgR) controls inhibitory responses induced by MAG. (A) Confocal microscope images of mouse DRG neurons showing increase in NgR levels, as detected by anti-NgR Abs and fluorescent secondary Abs, from embryonic day 17 (E17) to post-natal day 64 (adult). (B) Comparison of neurite outgrowth under control conditions containing IgG versus Fc-MAG, the extracellular domain of MAG fused to the Fc portion of IgG. Some experiments were carried out in the presence (+) or absence (−) of PI-PLC (to remove GPI-anchored proteins including NgR) or NgR-Ecto (truncated soluble NgR fragment that antagonizes Nogo-66). The asterisk denotes a statistically significant difference between MAG and control treated adult neurons, and there is no difference after interfering with NgRs. (C) Embryonic (E7) chick DRG explants were infected using recombinant virus to drive expression of green fluorescent protein (GFP) or NgR and treated with either GST control (glutathione S-transferase), GST-Nogo-66, IgG control, or Fc-MAG. (D) Quantitation of growth cone (GC) collapse shows that embryonic neurons are normally not responsive to MAG or Nogo-66, unless they are forced to express NgR (asterisks denote statistically significant differences). Reprinted with permission from Liu, Fournier, Grandpré, and Strittmatter, 2002, *Science* **297,** 1190–1193, 2002, American Association for the Advancement of Science.

adulthood (Fig. 17.11A). Second, MAG-mediated neurite outgrowth is eliminated after blockade of NgR operation (Fig. 17.11B). Several methods have been used in this and other studies described earlier to interfere with normal NgR activation, including (1) antibodies against NgRs, (2) soluble NgRs to compete for binding with neuronal receptors, (3) removal of NgRs by cleaving its GPI anchor, and (4) overexpression of truncated NgRs lacking the extreme C-terminal region (e.g., dominant-negative form of NgR), all of which prevent MAG/OMgP/Nogo-mediated inhibition. The last panel of Figure 17.11C shows that embryonic neurons, which are generally unresponsive to growth-inhibition by MAG and Nogo-66 proteins, become responsive when exogenous NgR is introduced into them. The assay involves measurement of growth cone collapse in DRG explants infected with recombinant virus to drive expression of green fluorescent protein alone or also NgRs. Similar experiments were used by Wang and coworkers to show that introduction of

exogenous NgRs confers OMgP responsiveness to otherwise insensitive neurons (Wang *et al*., 2002). Thus, the developmental switch in neuronal MAG sensitivity very likely involves an up-regulation of NgRs. Regardless, there is convincing evidence that NgRs are both necessary and sufficient for MAG-mediated growth inhibition in adult axons.

Is the interaction between NgRs and their multiple binding partners regulated? Although Nogo-66, OMgp, and MAG do not share any sequence similarity, binding studies show that they all bind to the NgR with very similar affinities (7, 5, and 8 nM, respectively). Figure 17.12 contains a model that illustrates the interactions of these three myelin-derived inhibitors with neuronal receptors including NgRs, as well as gangliosides and neurotrophin receptors, which will be discussed later. It is interesting that excess Nogo-66 does not prevent MAG-NgR binding (see Liu *et al*., 2002). Whereas MAG and Nogo-66 have separate binding sites within the same domain of NgR, OMgp and Nogo-66 have an overlapping binding site. If there is no preferential binding partner for NgRs, the particular membrane expression level for each NgR ligand may be an important factor that will determine the extent of axonal inhibition. Also intriguing is the fact that the MAG-NgR interaction is largely unaffected by the introduction of GT1b, a neuronal ganglioside that also interacts with MAG and will be covered in the next section. The MAG-NgR interaction may represent a more specific, biologically relevant interaction. Indeed, Nogo and MAG appear to be the main inhibitors in myelin, because when myelin is prepared from mice lacking Nogo-A, there is reduced inhibition of axon growth, and the residual activity is completely blocked by anti-MAG Abs (unpublished data; see Liu *et al*., 2002). It will be crucial to establish whether MAG, Nogo66, and OMgp bound to NgR and gangliosides all partake to regulate neurite outgrowth *in vivo* and whether complexes consisting of different combinations of these players can alter function.

The competition by multiple ligands for binding to the same receptor is a simple explanation for the redundancy observed when only a single ligand is blocked. The application of IN-1 Ab to block Nogo has only modest effects on improving axonal regeneration (Schnell and Schwab, 1990). Similarly, axons in MAG null mice do not show any significant improvement in regeneration (Li *et al*., 1996; Montag *et al*., 1994). Although there are no reports published on whether interfering with OMgp can improve axon outgrowth, a lack of significant effect is predicted. In contrast, a peptide antagonist of NgR (derived from a 40 amino-terminal fragment of Nogo-66, NEP1-40) is an effective blocker of myelin-mediated inhibition of axonal outgrowth and also significantly improves axon growth and functional recovery following spinal cord injury (GrandPre *et al*., 2002). Thus, the NgR appears to be a key player in the inhibitory actions of CNS myelin. This would be consistent with a model by which all three inhibitory ligands only exert their physiological inhibitory actions via one receptor, NgR. An obvious next step will be to generate a cocktail containing blockers of Nogo, MAG, OMgp, and also NgR in order to test whether this allows for superior axonal growth. The blockers will need to be designed to act in a transient function, because otherwise there may be excessive outgrowth and possibly an inability to form proper myelin. If this can be overcome, it should yield a potent theurapeutic agent that promotes functional recovery in many diseases and injuries affecting neurons.

Gangliosides Are MAG Receptors

The nerve cell surface gangliosides (sialic acid-containing glycosphingolipids) GD1a and GT1b are receptors for MAG (see the commentary by McKerracher, 2002). MAG binds sialic acid containing proteins and glycans, especially the NeuAc α3 Gal β3 GalNAc structure, which is commonly found on gangliosides (Collins *et al*., 1997b; Crocker *et al*., 1996; Kelm *et al*., 1994).

Binding studies indicate that GD1a and GT1b are the primary complex gangliosides in the brain that bind MAG (Collins *et al*., 1997a; Yang *et al*., 1996). Mice that lack the enzyme GM2/GD2 synthase, which is required for biosynthesis of complex gangliosides including GD1a and GT1b, display pathological features resembling those found in MAG knockouts (Sheikh *et al*., 1999). There is decreased CNS myelination and axonal degeneration in both CNS and PNS, and demyelination in the PNS. It should be noted that MAG

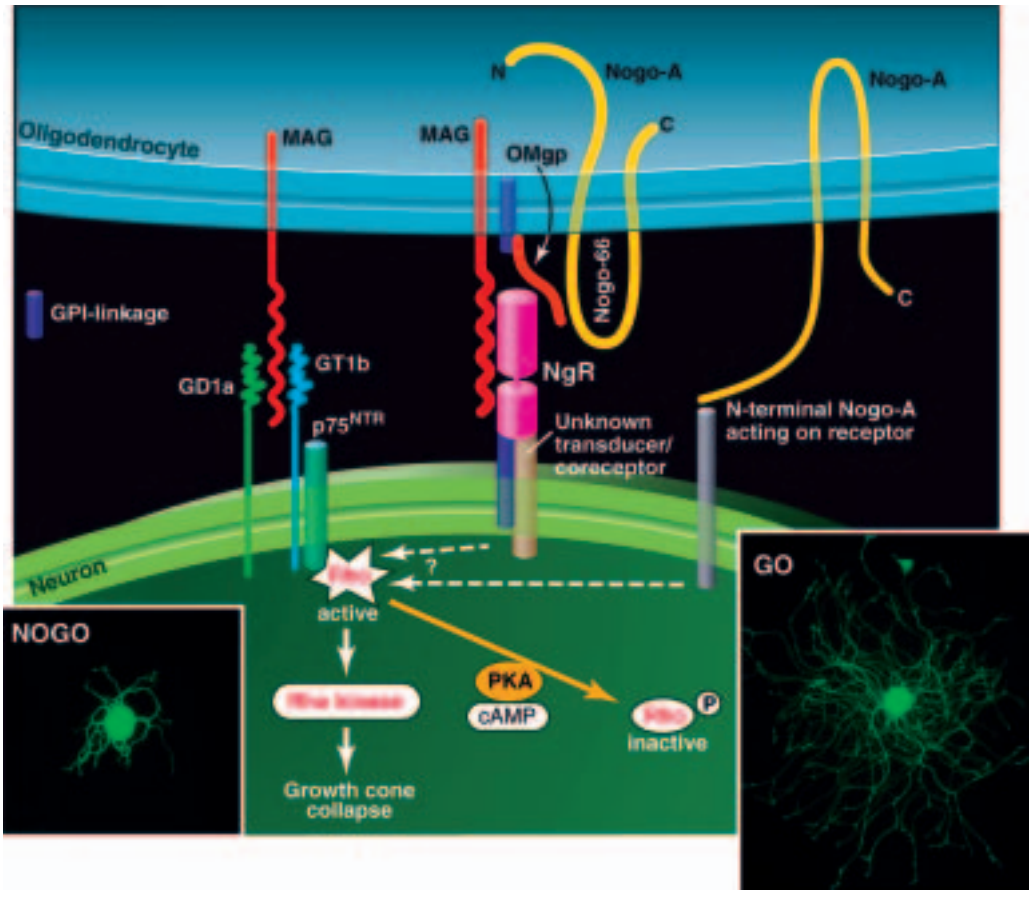

FIGURE 17.12
Model of MAG-mediated inhibiton of axon regeneration. Oligodendrocytes in the CNS produce three known growth-inhibitory molecules, MAG (red), OMgp (orange), and Nogo-A (yellow). The exact orientation of Nogo in the myelin membrane is not yet defined, however, both the N-terminal domain and Nogo-66 domain are inhibitory. MAG, OMgp and Nogo-66 all bind to the same neuronal receptor, NgR (Nogo receptor, pink), which results in blockade of neurite outgrowth and growth cone collapse. NgR lacks an intracellular domain and requires an unknown co-receptor to transduce intracellular signals via molecules such as Rho GTPase. MAG also binds to a complex consisting of gangliosides GD1a and GT1b, the latter of which interacts with the neurotrophin receptor $p75^{NTR}$. A signal for MAG-mediated inhibition of neuronal elongation is transduced through GT1b/$p75^{NTR}$ and activation of Rho. The photomicrographs show DRG neurons grown on a laminin substrate that is permissive for growth (right, GO) or on a CNS myelin substrate that prevents growth (left, NOGO). Reprinted with permission from Woolf and Bloechlinger, 2002, *Science* **297,** 1132–1133, 2002, American Association for the Advancement of Science.

expression is reduced by half in these GalNAcT −/− mice, although MAG heterozygote mice have similar reduction in MAG expression, yet they lack any neuropathology. Neurons from these GalNAcT knockout mice are less sensitive to MAG-mediated inhibition of neurite outgrowth (Vyas *et al*., 2002). Neurite outgrowth mediated by MAG is reduced upon addition of soluble GD1a and GT1b and also by Abs against GT1b, but not GD1a (Vinson *et al*., 2001). A subsequent report confirms the role of ganglioside GT1b in blocking neurite outgrowth and shows that anti-GD1a Ab is also effective (Vyas *et al*., 2002). Therefore, gangliosides GT1b and possibly GD1a regulate neurite outgrowth, and are neuronal receptors for MAG.

While evidence supports that MAG interacts with gangliosides, there is some debate as to whether gangliosides are necessary for MAG's inhibition of axonal outgrowth. For instance, removal of sialic acid residues does not affect binding of MAG to NgRs, receptors that mediate inhibition (Domeniconi *et al*., 2002). When MAG is mutated at arginine 118 (R118) to abolish sialic acid-dependent binding, MAG can no longer bind to neurons; however, when R118-mutated MAG is expressed on the surface of Schwann cells or CHO

cells, it is still a potent inhibitor of axonal outgrowth (Tang *et al.*, 1997a). These findings were interpreted to indicate sialic acid binding is insufficient to affect inhibition of axonal regeneration and that a second site on MAG must be responsible for this effect. Subsequent work suggested that the residual inhibition in the R118 mutant is dependent on sialic acid (Vinson *et al.*, 2001). Therefore, the R118-mutant MAG may have enough sialic acid binding to inhibit neurite outgrowth. However, there is additional evidence that sialic acid-dependent binding to neurons is insufficient to induce inhibition. Sialoadhesin (siglec-1) and a truncated form of MAG, MAG(d1-3)-Fc each recognize the same sialic acid linkage as MAG and also bind neurons in a sialic-dependent manner; however, none are inhibitory. Thus, there are probably multiple unique recognition sites on MAG, at least one of which binds sialoglycoproteins and another that mediates inhibition.

MAG may inhibit neurite outgrowth via multivalent clustering of gangliosides on neurons. Ganglioside-mediated inhibition of neurite outgrowth is independent of MAG because such inhibition can be induced in the absence of MAG by Ab-mediated clustering of GT1b (Vinson *et al.*, 2001; Vyas *et al.*, 2002). Compared to bivalent IgG Abs, pre-complexed Abs, and decavalent IgM Abs are more efficient in blocking axonal outgrowth in response to MAG. MAG binding to gangliosides may also function to cluster other signaling molecules, or even to regulate MAG activity, either of which could help potentiate inhibition of axonal growth (see Fig. 17.6).

Additional neuronal receptors may exist for MAG. For instance, although they are relatively scarce in brain, the α-series of gangliosides bind MAG with greater potencies than GD1a and GT1b (Collins *et al.*, 1999). However, the roles of α-gangliosides, if any, remain to be elucidated. In addition, De Bellard and Filbin have used MAG-Fc to precipitate several neuronal surface proteins (De Bellard and Filbin, 1999). The two most prominent proteins that bind MAG are of 190 and 250 kDa size, and the former is a sialoglycoprotein. Further work will need to further identify these potential MAG receptors as well as their actions.

SIGNALING EVENTS VIA MAG AND INTERACTING PROTEINS

Neuronal Signaling through MAG Receptors

Signaling via Neurotrophin Receptor, Rho GTPase, and cAMP

NgR lacks a transmembrane domain and is attached to the neuronal extracellular membrane by its GPI anchor. Without any cytoplasmic domains, intracellular signal transduction cannot occur. Thus, MAG (as well as Nogo-66 and OMgp) signaling through NgRs must be mediated via an associated transmembrane receptor. Similarly, gangliosides require signaling partners to transduce any signal across the cell membrane. The only known co-receptor is the low-affinity nonselective neurotrophin receptor p75 ($p75^{NTR}$), which binds GT1b (Yamashita *et al.*, 2002). MAG failed to inhibit outgrowth in neurons from mice carrying a mutation in the $p75^{NTR}$ gene. Further experiments on neurons from these mutant mice revealed that activation of the small GTPase Rho was lost. Finally, MAG fusion protein co-localizes with anti-$p75^{NTR}$ staining, suggesting they spatially interact. Hence, MAG inhibits neurite outgrowth via $p75^{NTR}$-mediated activation of Rho. It is not known whether MAG/NgR-mediated inhibition also operates by way of Rho signaling.

The $p75^{NTR}$-mediated signal was suspected to involve Rho because previous experiments had shown MAG activates Rho. Inactivation of Rho not only blocks growth inhibition by MAG and myelin, it also permits regeneration *in vivo* (Lehmann *et al.*, 1999). Neurons that cannot transduce Rho signals (expression of dominant-negative Rho) do not respond to MAG. Conversely, blockade of Rho-GTPase activity (using Clostridium botulinum C3 exoenzyme) allows neurons to grow on MAG substrates. An inhibitor of Rho kinase (Y27632), the downstream effector of Rho, blocks inhibition of neurite outgrowth induced either by myelin, MAG or anti-GT1b Ab (Dergham *et al.*, 2002; Vinson *et al.*, 2001). Furthermore, the latter study found Y27632 is of theurapeutic value because it promotes

axonal regeneration and functional recovery after spinal cord injury. To summarize, MAG binds a receptor complex consisting of ganglioside GT1b and $p75^{NTR}$, which serves as a signal transducer for neurite outgrowth inhibition via Rho and Rho kinase activation (see Fig. 17.12). It will be important to decipher the molecular participants in signaling events downstream of Rho, including activation of the Ser/Thr kinase known as Rho kinase. Another remaining issue is whether transduction of an inhibitory signal via $p75^{NTR}$ can be modulated according to whether $p75^{NTR}$ interacts with MAG or neurotrophins. Thus, $p75^{NTR}$ may act as a switch that permits outgrowth in environments containing neurotrophins, only to suppress outgrowth upon binding to MAG.

Neurotrophins and activation of the cAMP pathway can override MAG-mediated inhibition. Activation of $p75^{NTR}$ by the neurotrophin NGF abolishes Rho activation and enhances neurite elongation (Yamashita *et al.*, 1999). In addition, the neurotrophins BDNF and NT-3, which do not directly bind $p75^{NTR}$ but may interact via association with Trk receptors, can also stimulate neurite outgrowth. Priming of neurons by exposing them to BDNF or GDNF, or also by providing them with a cAMP analog, suspends the inhibitory actions of MAG on axonal outgrowth and regeneration (Cai *et al.*, 1999; Song *et al.*, 1998). It is speculated that MAG binding to neurons activates an inhibitory G-protein (Gi) that functions to block increases in cAMP and prevents growth. Introduction of cAMP analogs into spinal cord neurons allows them to regenerate (Neumann *et al.*, 2002; Qiu *et al.*, 2002). Interestingly, a reduction in endogenous levels of cAMP correlates with the developmental switch in neuronal response to MAG, which begins with promotion of neurite outgrowth in young neurons, and then changes to inhibiton (Cai *et al.*, 2001).

Do Rho and cAMP signals interact, and what effectors control neurite outgrowth? Elevation of cAMP activates protein kinase A (PKA), which subsequently phosphorylates Rho to induce its dissociation from the cell membrane (Lang *et al.*, 1996). This dual regulation of Rho activation and inactivation could act as a switch to control neuronal responses to MAG and neurotrophins. However, there are additional consequences of neurotrophin/cAMP signaling. Just lately, cAMP was shown to up-regulate Arginase I, an enzyme involved in the synthesis of polyamines (Cai *et al.*, 2002). After polyamine synthesis is blocked, BDNF and a cAMP analog can no longer suppress MAG- and myelin-mediated inhibition of neurite outgrowth. Either overexpression of Arginase I or priming with polyamines blocks inhibition mediated by MAG or myelin. Interestingly, the endogenous levels of Arginase I in DRG neurons drops during the first post-natal week and appears to coincide with the developmental switch from promotion to inhibition of neurite outgrowth in response to MAG. The authors discuss potential mechanisms by which polyamines can modulate neurite outgrowth and regeneration (also see the review by Skaper *et al.*, 2001). However, as discussed earlier, the developmental increase in NgR receptor also has been suggested to play a role in the post-natal switch in neuronal MAG sensitivity. It is possible that NgR and BDNF receptor signaling pathways interact to control neurite outgrowth. Future work will need to identify the interactions between downstream effectors of cAMP and Rho kinase. At the receptor level, it will be important to determine whether MAG-mediated signaling events through gangliosides and NgRs have any overlap or interaction. Such research will help in designing strategies that encourage axons to regrow in the CNS.

MAG Regulates Neuronal Cytoskeletal Proteins

In the section titled "Altered Morphology of Axons in MAG Null Mice," we noted that axons in MAG knockout mice have an altered morphology involving more densely packed neurofilaments (also see Fig. 17.10). Compared to wild-type mice, MAG-deficient mice older than 3 months of age have axonal profiles that are smaller with reduced spacing between neurofilaments (Yin *et al.*, 1998). The previously stated changes in axon caliber and cytoskeleton are likely a result of reduced neurofilament phosphorylation, which occurs in MAG knockout mice. This raises the possibility that changes in myelin are secondary to direct effects on axonal function. Similar alterations in axons and neurofilaments also occur in Trembler mice, which have a mutation in PMP-22 (de Waegh *et al.*, 1992). In the PMP-22 mutants, however, the alterations only occur in regions of sciatic nerve lacking myelin. MAG

knockouts have axonal defects even though myelin is present, suggesting that some other factor is responsible, such as altered signaling between glia and axons.

Neurofilaments consist of three subunits with low (NF-L), middle (NF-M), and high (NF-H) molecular weights, respectively. MAG-deficient mice have less NF-L and NF-H in nerve, while total NF-M is unchanged (Yin *et al*., 1998). Phosphorylation of NF-M and especially NF-H was significantly reduced (30 to 50% reduction depending on assay type). Phosphorylation of NF-H and NF-M increases the total negative charge and repositioning (lateral extension) of the neurofilament sidearms; this in turn normally functions to increase neurofilament spacing and axonal caliber. The mechanism of neurofilament arrangement has been analyzed using experimental data from wild-type and MAG-deficient mice and the most consistent model involves electrostatic repulsion between the phosphorylated sidearms (Kumar *et al*., 2002). Thus, under conditions of reduced NF-H and NF-M phosphorylation, the neurofilament sidearms retract, leading to reduced neurofilament spacing and smaller axonal calibers. In MAG-deficient mice, the diameter of the axons and neurofilament spacing are drastically reduced, particularly in paranodal regions. Whereas the paranodal region in wild-type mice continues to expand between P35 and P90, in MAG null mice the axon shrinks and this results in the formation of paranodal tomaculi. The schematic shown in Figure 17.10C illustrates the appearance of these abnormal paranodal myelin structures and also compares the structure of axons between wild-type and MAG null mice. Finally, it will be important to establish the signaling cascade that MAG initiates to control the neurofilament subunit composition and phosphorylation.

There is evidence that MAG interacts directly with microtubule-associated protein 1B (MAP1B) found on neurons. Although MAP1B is considered to be an intracelluar cytoskeletal component, it was proposed to also be a glycoprotein residing on the cell membrane (Muramoto *et al*., 1994). Recent analyses by Quarles and colleagues revealed that neurons express MAP1B, and that its carboxy terminus and glycosylation sites are located in the extracellular space, whereas the microtuble binding domains are in the neuronal cytoplasm (Franzen *et al*., 2001; Tanner *et al*., 2000). Although MAP1B is expressed by glial cells, a higher molecular weight isoform is expressed on neurons but not Schwann cells. Co-immunoprecipitation experiments show that MAG specifically interacts with MAP1B, and immunohistochemistry reveals they colocalize at axon-glia junctions both *in vitro* and *in vivo*. Moreover, when DRG neurons are cocultured with MAG-expressing COS cells, there is a two-fold increase in expression of phosphorylated MAP1B, as well as increases in NF-H and NF-M, which are described in the next paragraph. These MAG-mediated cytoskeletal phosphorylation events are important for control of axon caliber, as demonstrated by the axonal changes that occur in MAG nulls when such signaling is missing. Similarly, neurofilaments in peripheral nerves of Trembler mutants have reduced phosphorylation and increased density, and interestingly there is less MAP1B phosphorylation (Kirkpatrick and Brady, 1994). Shiverer mice have a natural mutation, which yields a truncated form of MBP, and their CNS axon microtubule expression and density is increased (Kirkpatrick *et al*., 2001). The signaling mechanisms responsible for altering the axonal cytoskeleton in these mutants are not known, but potentially involve changes in MAG expression, which occur in knockouts (see Tab. 17.1). Perhaps most intriguing is the potential for MAG-MAP1B interactions to provide a structural link between axons and glia, and directly contribute to the modulation of myelinated axon structure and stability.

The ability of MAG to regulate the axonal cytoskeleton has been demonstrated in cultured neurons and has also been extended to expose the underlying mechanisms. Compared to co-culturing of DRG neurons with control COS cells, co-culturing with MAG-expressing COS cells results in a five- and two-fold increase in NF-H and NF-M phosphorylation, respectively (Dashiell *et al*., 2002). MAG also causes the level of NF-M expression to increase by 50%. Treatment of PC12 neurons with soluble MAG-Fc chimera also effectively increases NF-M expression and phosphorylation, thus demonstrating that MAG is responsible for the axonal changes, rather than other mechanisms such as cell-cell interactions. MAG also increases MAP1B, phosphorylated MAP1B, MAP2, low-molecular-weight tau, and growth-associated protein-43 (GAP-43) levels.

Whole extracts of sciatic nerves from MAG null mice were also studied to determine whether the reduced neurofilament phosphorylation in MAG null mice is due to altered kinase activity (Dashiell *et al*., 2002). Based on an assay that evaluates phosphorylation of preferred kinase substrates, MAG knockout mice had reduced activity of proline-directed kinases, including cyclin-dependent kinase-5 (cdk5) and extracellular-signal regulated kinases 1 and 2 (ERK1/2). The specific activities of the cdk5 and ERK1/2 enzymes were subsequently determined to be reduced by 33 and 38%, respectively. Kinase assays also demonstrate that when these enzymes are immunoprecipitated from PC12 neurons treated with MAG-Fc, their activity is significantly increased. In summary, MAG activates cdk5 and ERK1/2 kinases and this signaling process may be involved in MAG-mediated regulation of the axonal cytoskeleton. Further work will need to determine whether these or other kinases are obligatory, and whether MAG modulates phosphatase activities, transcription, and degradation of cytoskeletal proteins.

Glial Signaling via MAG

The cytoplasmic domains of MAG may be involved in modulating MAG function or signaling through phosphorylation, association with kinases, and also the cytoskeleton. Several interesting signaling events that may be of biological relevance to myelin formation occur within minutes of Ab-induced cross-linking of L-MAG. First, Fyn kinase activity increases, which leads to phosphorylation of L-MAG on Tyr620 (Jaramillo *et al*., 1994; Umemori *et al*., 1994). Consequently, this phosphorylation event allows MAG to associate with phospholipase Cγ (PLCγ). Nonreceptor tyrosine kinases such as Fyn and PLC associate via their SH2 and SH3 domains with specific sequences containing a phosphorylated Tyr in the cytoplasmic domain of surface receptors and CAMs. Thus, MAG may serve as a docking protein, which allows interactions between different signaling molecules. Fyn activation by MAG may be of great importance because myelination is partially impaired in fyn-deficient mice. MAG interaction with neuronal receptors could trigger events such as myelin formation.

The regulation of phosphorylation events may be complex, and it cannot be excluded that other signaling events through separate receptors and signaling pathways can feed back onto MAG. We suggested previously that the phosphorylation state of the MAG cytoplasmic domain modulates adhesion and wrapping of the glial cell membrane around the axon during the myelination process (see the review by Attia *et al*., 1989). The strength of the MAG-neuron interaction could be regulated differentially between the leading glial lip and the previous myelin wrap by phosphorylation. In addition to controlling adhesion, the regulation of kinase and phosphatase activities may be responsible for initiating and terminating myelin wrapping.

Besides interactions with kinases, MAG binds other cytoplasmic proteins. There is evidence that S100β, a 10 kDa calcium- and zinc-binding protein, can bind to the cytoplasmic domain of L-MAG (Kursula *et al*., 1999). A S100β synthetic peptide corresponding to the L-MAG interacting cytoplasmic domain (putative basic amphipathic alpha-helix) reproduced this interaction including the Ca^{2+}-dependency and further revealed a binding stoichiometry of 1:1 with a dissociation constant (K_D) of 7 μM. This represents a fairly strong interaction that could also take place *in vivo* and partially contribute to MAG-mediated signaling. Another interesting finding is that in the presence of S100β, phosphorylation of the L-MAG cytoplasmic domain by PKA is inhibited (Kursula *et al*., 2000). The functional consequences of S100β-mediated inhibition of phosphorylation is being investigated. However, S100β interacts with a variety of cytoskeleton-associated proteins, including actin microfilaments, microtubules, and intermediate filaments. Thus, MAG-mediated control of glial cell morphology and function may be modified by interactions with S100β.

Finally, there is some progress regarding identification of glial cytoskeletal components that interact with MAG. A potential cytoskeleton binding site, which is similar in sequence to a functional site in the β1 subunit of integrin, is found in both isoforms (Salzer *et al*.,

1987). Co-localization of MAG and cytoskeletal components such as F-actin and spectrin is consistent with this prediction (Trapp *et al*., 1989b).

MAG IN INJURY AND DISEASE

Human Gene Mutations in MAG?

Based on studies of MAG knockout mice, it is probable that human diseases that affect only MAG will remain elusive. Analyses of MAG single knockout mice have failed to show defects in myelin formation, and instead, it is the long-term maintenance of myelin that is affected (Fruttiger *et al*., 1995a; Yin *et al*., 1998). No electrophysiological defects in peripheral nerve conduction occur in 4- to 8-week-old MAG-deficient mice (Montag *et al*., 1994), and most behavioral motor and posture patterns are normal in the open field; however, the bar-cross apparatus that tests for fine motor coordination revealed several abnormalities (Li *et al*., 1994). MAG nulls have decreased locomotor activity, frequency of exploratory sniffing and grooming, and a mild, transient, trunk tremor on the bars (see Tab. 17-1 for a summary). Similarly, another study found that compared to wild-type mice, only half of the MAG-deficient mice were able to cross a horizontal bridge and there was an increased latency to reach a platform, whereas open field behavior was only mildly affected (Uschkureit *et al*., 2000). Thus, there are no gross abnormalities in MAG mutants; however, their finer motor coordination abilities are impaired. Aging mice do reveal neurological dysfunctions in that there is a significant decrease in conduction velocity, an impairment that also occurs in human demyelinating neuropathies (Weiss *et al*., 2001). On the other hand, at least seven other action potential parameters were unchanged, again demonstrating that the defects are minor. Given that the neuropathology in MAG knockout mice is not severe, and together with the ability to only detect it easily later in life, this suggests that MAG mutations in humans would be difficult to diagnose.

As far as we are aware, there is no unequivocal evidence for mutations in MAG as contributing to human disease. Although there was some suggestion that genes influencing MS reside within or close to human chromosome 19q13, genetic linkage analyses indicate that MAG is not a leading candidate gene (D'Alfonso *et al*., 2002). Instead, the apolipoprotein E gene, which resides in the same chromosomal region, is more favored to play a role (Schmidt *et al*., 2002). Still, this locus confers only a part of the genetic susceptibility in MS. However, MAG mutations may play a role in other neuropathies, and further linkage studies will be necessary to determine whether the MAG gene is a candidate. It is also conceivable that alterations in other genes can affect MAG transcription, such as in the abnormal QKI expression found in qk mice. Thus, future studies will need to consider mutations that are affecting transcription or post-translational modification of MAG.

Autoimmune Neuropathy

Auto-Abs to MAG and also gangliosides are often associated with immune-mediated paraproteinemia and peripheral neuropathies (see reviews by Quarles and Weiss, 1999; Steck *et al*., 1999). When associated with MAG auto-Ab, the neuropathies involve neuronal or axonal degeneration or demyelination, the condition is chronic, and it affects both sensory and motor nerves. There are many clinical studies describing the presence of anti-MAG Abs in neuropathies, although they cannot all be referenced here (for example, see Braun *et al*., 1982; Melmed *et al*., 1983). For neuropathies associated with monoclonal gammopathy, MAG is the most commonly targeted antigen. In a clinical study of 15 patients with a demyelinating sensory polyneuropathy, most had Abs to MAG (Van den Berg *et al*., 1996). Larger studies (40 to 75 patients) involving neuropathies associated with IgM gammopathy revealed 56 to 65% had anti-MAG monoclonal Abs (Chassande *et al*., 1998; Nobile-Orazio *et al*., 1994;). From other studies, it is

known that the MAG auto-Abs commonly react with the HNK-1 epitope, although there are differences in specificities with regard to binding sulfate and carboxlic acid moieties, and there are also differences between patients in the Ab binding strength (Ilyas *et al*., 1984, 1990; Weiss *et al*., 1999; Yeung *et al*., 1991). In demyelinating neuropathies, the Ab titers to MAG appear to correlate with the degree of demyelination observed by histology and electrophysiology (Chassande *et al*., 1998; Gabriel *et al*., 1996). The serum anti-MAG titres correlate inversely with the amount of detectable MAG in nerve biopsies from the same patients, even though other myelin-related proteins such as P0, MBP, and periaxin are unaffected.

MAG influences the structure and biochemistry of axons in patients that have demyelinating neuropathies with MAG auto-Abs present. Neurofilament phosphorylation and spacing is reduced in MAG null mice (Yin *et al*., 1998), and similar changes occur in human patients with MAG auto-Ab-related demyelinating neuropathies (Lunn *et al*., 2002). Electron microscopy reveals that neurofilaments from diseased human sural nerve axons are more closely spaced in those patients that have anti-MAG auto-Abs. Axons from normal subjects and also demyelinated axons from patients with other neuropathies are not affected, demonstrating that the effect is specific to patients with MAG auto-Abs. There is a nonsignificant trend for diseased axons to be smaller in diameter. Axonal constriction may account for the reduced conduction velocity observed in aged MAG null mice. A loss of signaling between Schwann cells and axons followed by decreased neurofilament phosphorylation is postulated to be responsible for the axonal changes in MAG null mice and in patients with MAG auto-Abs.

Data support the notion that the auto-Abs are indeed targeting MAG on glial cells. First there is biochemical evidence based on western blotting and also by the ability of auto-Abs to bind MAG peptides. Furthermore, immunocytochemical data reveal that MAG auto-Abs from patient serum binds to myelinating Schwann cells (Takatsu *et al*., 1985). Prior absorption of serum with MAG abolishes staining. Anti-MAG IgM deposits occur at sites of uncompacted myelin including Schmidt Lanterman incisures and paranodal loops (Gabriel *et al*., 1998; Steck *et al*., 1999). Furthermore, Abs bind to the Schwann cell basal lamina of myelinated (but not unmyelinated) fibers and are co-localized with collagen IV. In summary, auto-Abs to MAG are associated with demyelination, their abundance is related to the severity of the neuropathy, and they bind to myelinating Schwann cells.

There is also an indication that in several diseases of the nervous system, anti-ganglioside Abs are involved (see the review by Takamiya *et al*., 1996). A subset of neuropathies are associated with auto-Abs to GD1a and GT1b, which bind MAG. Patients with demyelinating neuropathy have circulating Abs against GD1a, GT1b, and GM3, the latter of which also binds MAG although with reduced potency (Mizutani *et al*., 2001; Vabnick *et al*., 1997). In a study of 195 patients having an assortment of motor syndromes, high anti-GD1a Ab titers were detected in two patients with IgM monoclonal gammopathy and two patients with Guillain-Barré syndrome (Carpo *et al*., 1996). The latter disease is an acute demyelinating inflammatory polyneuropathy often associated with auto-Abs to gangliosides (including GD1a), and evidence is accumulating to suggest an immune response to an environmental immunogen is involved (see reviews by Quarles and Weiss, 1999; Nachamkin *et al*., 2002; Schwerer, 2002). In this case, molecular mimicry occurs from Campylobacter jejuni bacterial infection, which presents an antigen similar to human gangliosides.

Gangliosides and MAG auto-Abs may also play a role in a variety of other diseases, including MS, optic neuritis, and myasthenia gravis (for example, see Link *et al*., 1992; Möller *et al*., 1987; Sato *et al*., 1986; Wajgt and Gorny, 1983). Among 25 MS patients, 15 had anti-MAG Abs (12 IgG, 3 IgM) in their CNS fluid, compared to only 1 out 27 control patients (Baig *et al*., 1991). A study of 32 MS and 20 optic neuritis patients found 23% and 18%, respectively, had anti-GD1a Abs (Mata *et al*., 1999). Thus, there is clinical evidence that gangliosides which bind to MAG are involved in neurological disease.

While auto-Abs can induce demyelination, the reasons for their production in neuropathies is unclear. There is sufficient evidence that anti-MAG and anti-GD1a Abs can induce axonal demyelination and degeneration. Injection of cats, rabbits, or chickens with anti-MAG Abs causes demyelination (Hays *et al*., 1987; Tatum, 1993; Trojaborg *et al*., 1989; Willison *et al*., 1988). The ultrastructural pathology in these animal experiments is very similar to that found in human neuropathy, including the widening of myelin lamellae. Mice exposed to anti-GD1a Abs develop axonal degeneration (see the report by Willison *et al*., 2002). Regarding neuropathologies, the trigger for auto-Ab production by T cells could be antigens that enter the circulation from damaged myelin. Damage to the myelin sheath releases the soluble proteolytic fragment of MAG, dMAG, which inhibits axonal regeneration (Tang *et al*., 1997b, 2001) and has been suggested to contribute to neurological disease (Johnson *et al*., 1986). A study of five MS patients found that the dMAG form was more abundant than in control subjects and seemed to account for the preferential loss of MAG at MS plaques (Möller *et al*., 1987). Another study found that lymphocytes in patients with MS and polyneuropathy responded to MAG and to five synthetic MAG peptides to a greater extent relative to healthy subjects (Andersson *et al*., 2002). However, since none of the peptides appear to be immunodominant, the authors conclude that MAG auto-Abs arise through secondary responses associated with myelin breakdown. However, they did not exclude a possibility that these Abs play a role in the initiation or progression of the diseases. Since there is no evidence for molecular mimicry with a MAG-like epitope, an immune response to endogenous myelin does appear to be a simple reason why MAG is targeted so frequently in a variety of neurological disorders, particularly those involving demyelination. Finally, it is not known whether the pathogenic effects of MAG Abs are moderated by disruption of normal MAG function or also by cell death through complement-mediated lysis.

SUMMARY AND CLOSING REMARKS

MAG is a glycoprotein localized in the periaxonal membranes of glia that form the specialized wrappings known as myelin. Evidence supports multiple functional roles for MAG, including the formation and maintenance of myelin. MAG also affects axonal viability and cytoskeletal organization, and recent studies show that MAG inhibits axonal sprouting and regeneration. Regarding this latter role, a wealth of information recently became available including the remarkable discovery that MAG, Nogo, and OMgp all bind to NgR. This finding explains why removal of only some of the myelin-derived inhibitory molecules is not sufficient to block inhibition mediated by myelin from the adult CNS. Furthermore, the absence of NgR during early development appears to explain why these myelin-derived molecules do not inhibit young neurons. The identification of NgR, together with additional ganglioside receptors for MAG, now makes it possible to focus on and further characterize MAG-mediated molecular interactions and signals. Inhibition of neurite outgrowth by way of MAG/NgR signaling may either overlap or be distinct from ganglioside/$p75^{NTR}$-mediated intracellular signaling events involving Rho kinase. It is now possible to determine whether these molecular events also apply to processes involving the genesis and stability of myelin.

Studies of transgenic mice have revealed important details regarding MAG function. For instance, the L- and S-MAG isoforms have different functions, the former being crucial for formation of CNS myelin and the latter contributing to maintenance of PNS myelin. Mice lacking MAG entirely have mild defects in the formation and maintenance of myelin. Myelination is delayed, superfluous myelin is formed, there is increased degeneration of myelin and axons, and both the ultrastructure and biochemistry of axons are altered. The defects are not prominent, and superficially it may seem that MAG does not have an essential role. However, there is significant molecular redundancy in the nervous system, and it is difficult to appreciate the full contribution of endogenous MAG function.

For instance, the role of MAG in the spiraling process of myelination only becomes apparent in knockout mice lacking both MAG and P0.

Behavioral and electrophysiological changes in MAG-deficient mice present a phenotype similar to that found in human neuropathies and demyelinating diseases. MAG and its ganglioside receptors are a frequent target of auto-Abs in immune-mediated neuropathies. Mice exposed to anti-MAG Abs develop axonal degeneration and demyelination, and the pathology is similar in human patients with MAG auto-Abs. However, there is no human disease that has been mapped to the MAG gene, possibly because the phenotype is not severe in younger individuals and thus is difficult to detect.

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